Molecular Cloning and Analysis of the frd-DNA Ligase Region of the Genome of Bacteriophage T4

by

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Foreword

Apart from the isolation of some of the λ*td* phages generated by R.*Eco*RI and polyacrylamide gel analyses of polypeptides, which were done in collaboration with Dr Helen Revel, the work presented in this thesis has been my own. Many of the approaches and ideas contained within this work were devised in discussions with my supervisor, Dr Noreen Murray.

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Most of all thanks to Karen, who not only provided many enzymes, helped at vital stages in the work, proof read and gave me support when things went wrong, but also became my wife during the course of this work.

Abstract

In vitro recombinants of bacteriophages λ and T4, containing the T4 gene for thymidylate synthetase (*td*), have been isolated by their ability to complement the *E.coli thyA* gene. These λ derivatives, together with λ hybrids carrying the T4 DNA ligase gene (g30), have facilitated the cloning of nearly all the DNA between genes 30 and *td*, a region of the T4 genome that includes two genes coding for products useful in biochemical research (RNA ligase (g63) and polynucleotide kinase (*pseT*)).

A restriction map of the dihydrofolate reductase (*frd*)-DNA ligase region of the T4 genome has been constructed using several restriction endonucleases. Gene expression, complementation, marker rescue and hybridisation studies, have defined the positions of several genes within this map and facilitate the isolation of λ derivatives carrying functional copies of genes coding for polynucleotide kinase and RNA ligase.

Abbreviations and Conventions

kb - kilobase

gX - gene X

gpX - protein product of gene X

UV - ultraviolet light

mM - milli-Molar

HMC - Hydroxymethylcytosine

K - Kilodalton

1) Genetic nomenclature for E.coli according to Bachmann, B.J., Low, K.B. and Taylor, A.L. (1976) Bacteriol.Rev. 40, 116-167; and for T4 according to Wood and Revel (1976). Genotypes will be indicated by underlining the gene symbol.
2) In general bacterial and phage strains will be referred to by their key genotype.
3) Restriction endonuclease nomenclature according to Smith, H.O. and Nathans, D. (1973) J.Mol.Biol. 81, 419-423
4) Orientation of genes in a λ recombinant is defined according to their direction of transcription. l orientation denoted leftward, and r orientation, righward transcription.

CONTENTS

1. INTRODUCTION

Bacteriophage T4

T4 was amongst the earliest bacteriophages to be discovered (Demerec and Fano, 1945) and has since become the most intensely studied large virulent bacteriophage (see Wood and Revel, 1976). It has also been the subject of many classical genetic studies (e.g. Benzer, 1959, 1961; Crick et al, 1961).

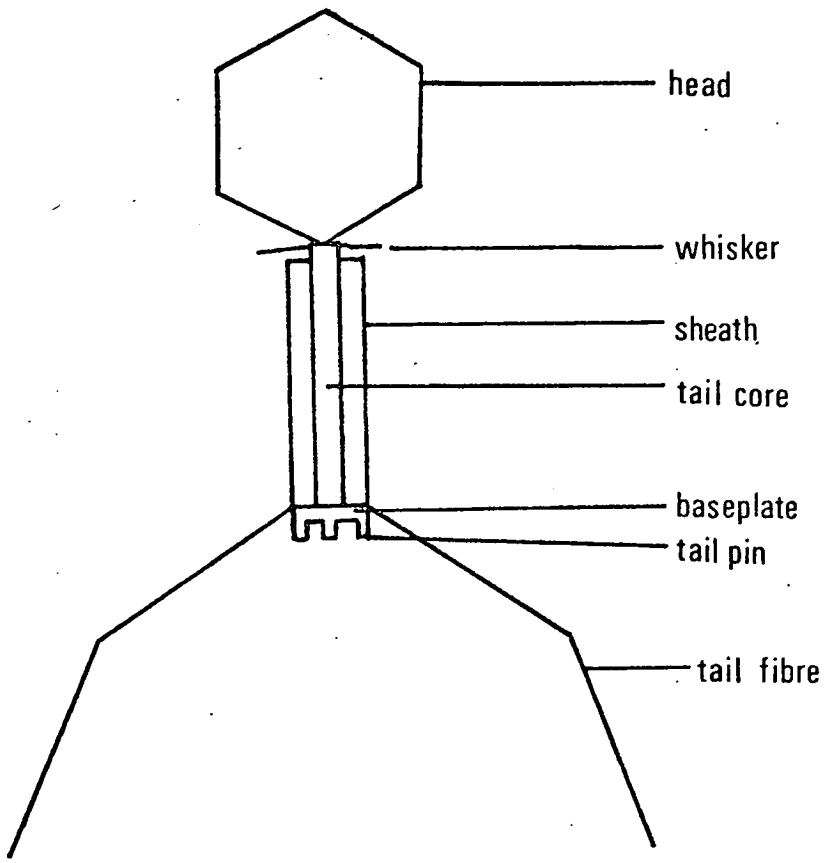
T4 is a member of the T-even group of phages which also includes T2 and T6. These are large DNA viruses whose complex morphology is necessary for their mode of infection (see Figure 1.1 for a diagram of phage T4). Their genomes are basically homologous and so T-even phage can undergo genetic recombination freely with each other. They also display complementation ability and serological cross reactivity. Heteroduplex studies have revealed that T-even phage have about 85% nucleotide sequence homology with each other, the greatest diversity being in the genes specifying host range (Kim and Davidson, 1974). The gene products involved in host recognition include the tail fibres which are serologically distinct within the group. Indeed the greatest degree of heterology shown by heteroduplex studies is in the tail fibre region around genes 37 and 38 (see Figure 1.2 for a map of the T4 chromosome).

T-even DNA is circularly permuted, thus although the DNA of the phage particle is linear, the genetic map is circular (Streisinger et al, 1964). Mature phage DNA also displays terminal redundancy (Thomas and Rubenstein, 1964), which ensures that an entire genome is always packaged. T-even phage package their DNA by a 'headful' mechanism and compensate for deletions of non-essential regions of the genome by increasing the length of their terminal

Figure 1.1

Schematic diagram of the T4 phage particle.

Figure 1.1


head
whisker
sheath
tail core
baseplate
tail pin
tail fibre

repetitions (Streisinger et al, 1967). Mature T-even heads contain 170 kb of DNA, although the genome size varies from 160 to 166 kb amongst the group (Kim and Davidson, 1974). T-even DNA is packaged from concatemeric DNA (Ritchie and White, 1972) which, together with the method of packaging, explains both the presence of terminal redundancy and the circularity of the genetic map.

T-even DNA is unusual in that it contains hydroxymethycytosine (HMC) instead of cytosine (Wyatt and Cohen, 1953) and these residues are glucosylated either at the α or β position (Lehmann and Pratt, 1960). Some adenine residues are also methylated (Hattman, 1970).

T4 has been the main focus of attention of research involving T-even phage, but it is almost certain that principles revealed for T4 will also apply to T2 and T6.

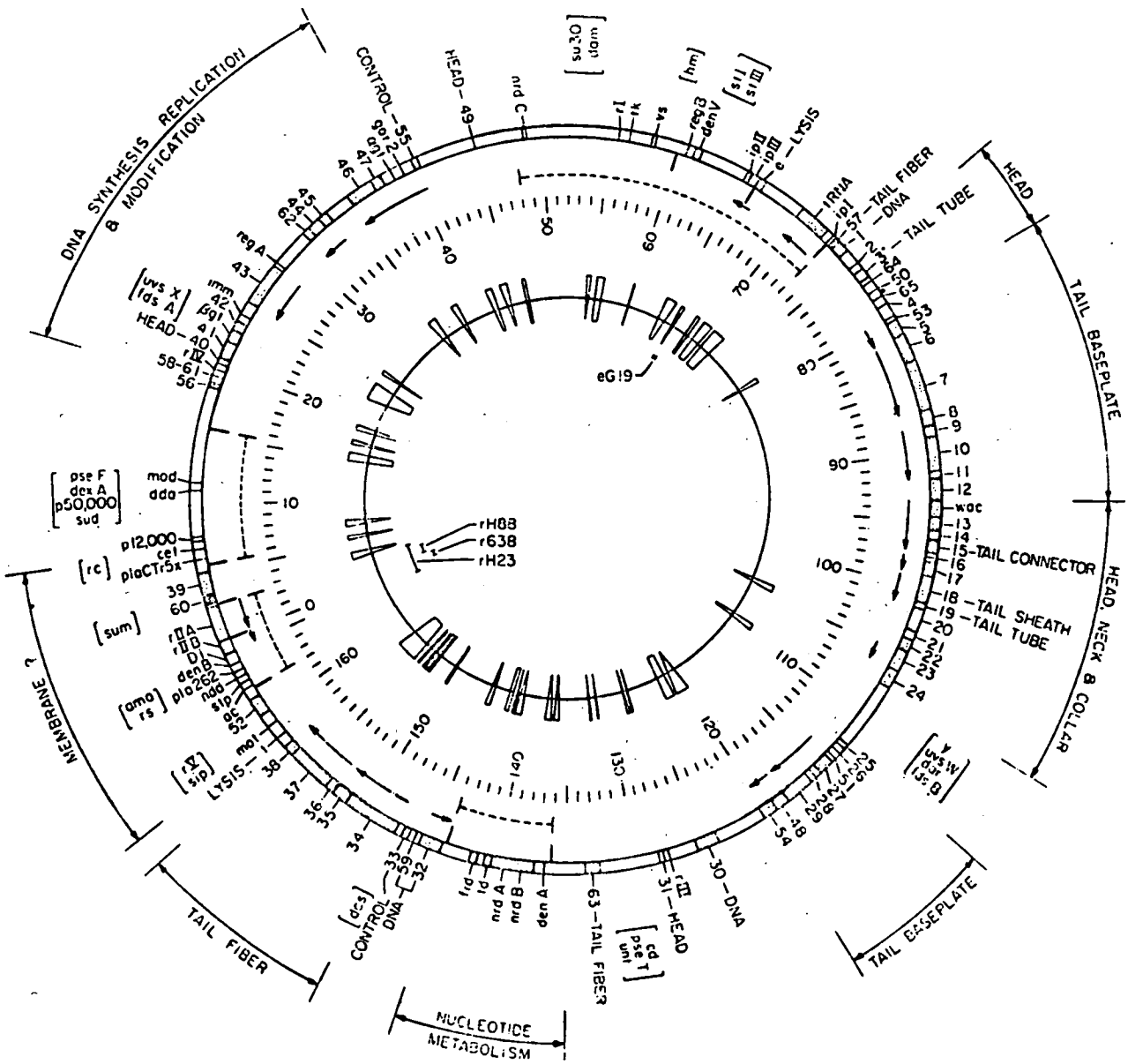
Gene Classes and Designations

T4 genes are divided into two groups, the essential and non-essential genes. Essential genes, designated mainly by numbers, and seldomly by lower case single letters, are those that show conditionally lethal mutations (Epstein et al, 1963), whereas non-essential genes, designated by two or three letter symbols, are those that do not show lethal mutations on normal laboratory strains. However, some non-essential gene mutations have quantitative effects on phage multiplication under certain conditions or on certain bacterial strains (e.g. Sirotkin et al, 1978) and can even be conditionally lethal (e.g. Mattson et al, 1979). This implies that non-essential genes augment already existing host functions to optimise the level or quality of certain molecules during infection.

T4 genes are also classified by the time of appearance of their products into immediate-early (IE), delayed-early (DE), quasi-late and true-late genes (O'Farrell and Gold, 1973).

Figure 1.2

Genetic map of T4, taken from Wood and Revel (1976). The innermost circle shows the regions of non-homology between T4 and T2 (Kim and Davidson, 1974), and the laballed arcs are deletions used as references. The scale is in kilobase units from an arbitrary zero at the rIIA-rIIB join. Arrows indicate the direction of transcription of genes covered by the arrow: those covering more than one gene indicate co-transcription. Dashed lines indicate the extent of non-essential regions defined by overlapping deletions in viable phages. Bars on the map circle represent the minimum length of particular genes and gene names are shown outside this circle; gene names in brackets represent loci whose position on the map is only approximate. The outermost circle indicates the functional clustering of genes.


DNA SYNTHESIS REPLICATION & MODIFICATION
CONTROL
HEAD
HEAD
TAIL BASEPLATE
HEAD, NECK & COLLAR
TAIL TUBE
DNA
TAIL FIBER
TAIL CONNECTOR
TAIL SHEATH
TAIL TUBE
TAIL BASEPLATE
MEMBRANE ?
TAIL FIBER
LYSIS
CONTROL
DNA
NUCLEOTIDE METABOLISM
TAIL FIBER
HEAD
DNA
eG19
rH88
r638
rH23

The T4 Life Cycle

(a) Infection

The tail fibres of non-infective phage particles are wrapped around the phage tail and held at the joint between the phage head and tail by the tail whiskers which are the products of the T4 *wac* gene (Wood and Conley, 1979; see Figure 1.1). L-tryptophan released from bacterial cells induces the unwrapping of the tail fibres (Conley and Wood, 1975), and the tips of the tail fibres then interact with the lipopolysaccharide molecules of the bacterial outer cell wall (Wilson *et al*, 1970; Beckendorf *et al*, 1973). A contraction of the tail fibres brings the tail base plate into contact with the cell wall where the base plate pins bind firmly This induces a conformational change in the base plate, and event which is followed by the contraction of the tail sheath. The binding of the base plate and contraction of the sheath, forces the tail core to penetrate into the cytoplasm of the cell. These events have been visualised by electron microscopy (Simon and Anderson, 1967). The base plates of T-even phage possess a lysozyme activity which digests the peptidoglycan of the cell wall and facilitates penetration. This activity is supplied by the normal endolysis in T2 infections (Koch and Dreyer, 1958) and by gp5 in T4 infections (Kao and McClain, 1980).

Penetration of the tail tube into the host cytoplasm triggers the injection of the phage DNA, and certain other molecules, into the host cell.

(b) Shut off of host transcription

T4 infection results in the complete inhibition of host gene expression. This is effected at the post-transcriptional as well

as the transcriptional level. Although host mRNA can be both initiated and elongated for several minutes after infection, there is an immediate block on the induction of host specific enzymes such as β-galactosidase and hybridisation studies show that lac mRNA, in these conditions, is not associated with ribosomes (Kennel, 1970). This effect also occurs in the presence of rifampicin and since it occurs immediately upon infection, shut off here must be due to a preformed T4 product. During early infection the host RNA polymerase is modified at several stages, so that it will finally only transcribe from an HMC containing template (see sections (e)iii and (j)).

(c) Breakdown of host DNA

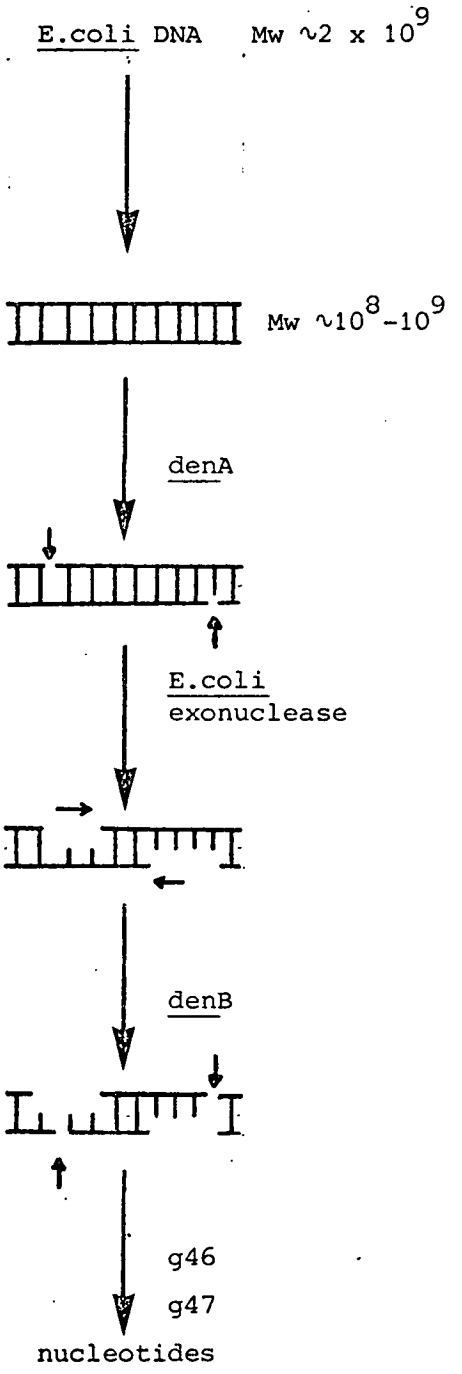
T4 produces a battery of nucleases, that specifically degrade cytosine containing DNA, at early times after infection. The product of the denA gene, endonuclease II, acts on double stranded E.coli DNA to produce nicks at the 5' side of cytosine residues (Sadowski and Hurwitz, 1969). An exonuclease activity, probably a host function, digests the nicked strand in a 3'-5' direction producing single stranded regions. The product of the phage denB gene, endonuclease IV, then fragments the host DNA by cutting to the 5' side of cytosine residues in single stranded regions (Sadowski and Bakyta, 1972). A phage encoded exonuclease, probably gps 46 and 47, finally degrades these fragments to mononucleotides (see Warner et al, 1970). These reactions are summarised in Figure 1.3.

As any cytosine containing DNA in the infected cell is quickly degraded, T4 avoids prolonged transcriptional competition with the host and enriches the intracellular nucleotide pool.

Figure 1.3

Breakdown of host DNA in T4 infected cells. The T4 genes whose products are involved in this process are indicated above the arrows. Short, black headed arrows indicate positions of endonuclease cuts, and white headed arrows, the direction of exonuclease digestion. Based on Warner *et al* (1970).

Figure 1.3


E.coli DNA Mw ∿2 x 10^9
Mw ∿10^8-10^9
denA
E.coli
exonuclease
denB
g46
g47
nucleotides

(d) Nucleotide metabolism in infected cells

T4 DNA codes for several enzymes involved with nucleotide metabolism, and these include the products of both essential and non-essential genes. Host DNA degradation in T4 infected cells results in the accumulation of a pool of mononucleotides that can be channelled into T4 DNA replication. The conversion of cytosine to HMC occurs at the mononucleotide level. gp 56, a potent deoxycytidine-deoxyuridine di- and tri-phosphatase (Wiberg, 1966) essentially ensures that the only cytidine phosphate derivative in the cell is dCMP, thus cytosine cannot be incorporated into T4 DNA. gp 42 is a deoxycytidylate hydroxymethylase which converts dCMP to dHMCMP, the methyl group being transferred from methylene tetrahydrofolate (Dirksen et al, 1963). All deoxyribonucleotide monophosphates are converted to deoxyribonucleotide triphosphates by the action of gp 1, a deoxyribonucleotide kinase, and thus made available for DNA synthesis (Duckworth and Bessman, 1967). The T4 genome also codes for several non-essential components involved in nucleotide metabolism including ribonucleoside diphosphate reductase, gps nrdA and nrdB (Yeh and Tessmann, 1972), a thioredoxin, gp nrdC (Tessmann and Greenberg, 1972), a deoxycytidylate deaminase, gp cd (Hall et al, 1967), a thymidylate synthetase, gp td (Shapiro et al, 1965), a dihydrofolate reductase, gp frd (Hall, 1967) and a thymidine kinase, gp tk (Chace and Hall, 1975a). These reactions are summarised in Figure 1.4. T4 also codes for many enzymes involved in nucleic acid metabolism including a DNA ligase (gp 30) (Fareed and Richardson, 1967) and a polynucleotide kinase (gp pseT) (Sirotkin et al, 1978).

The genes coding for these enzymes show a degree of clustering,

Figure 1.4

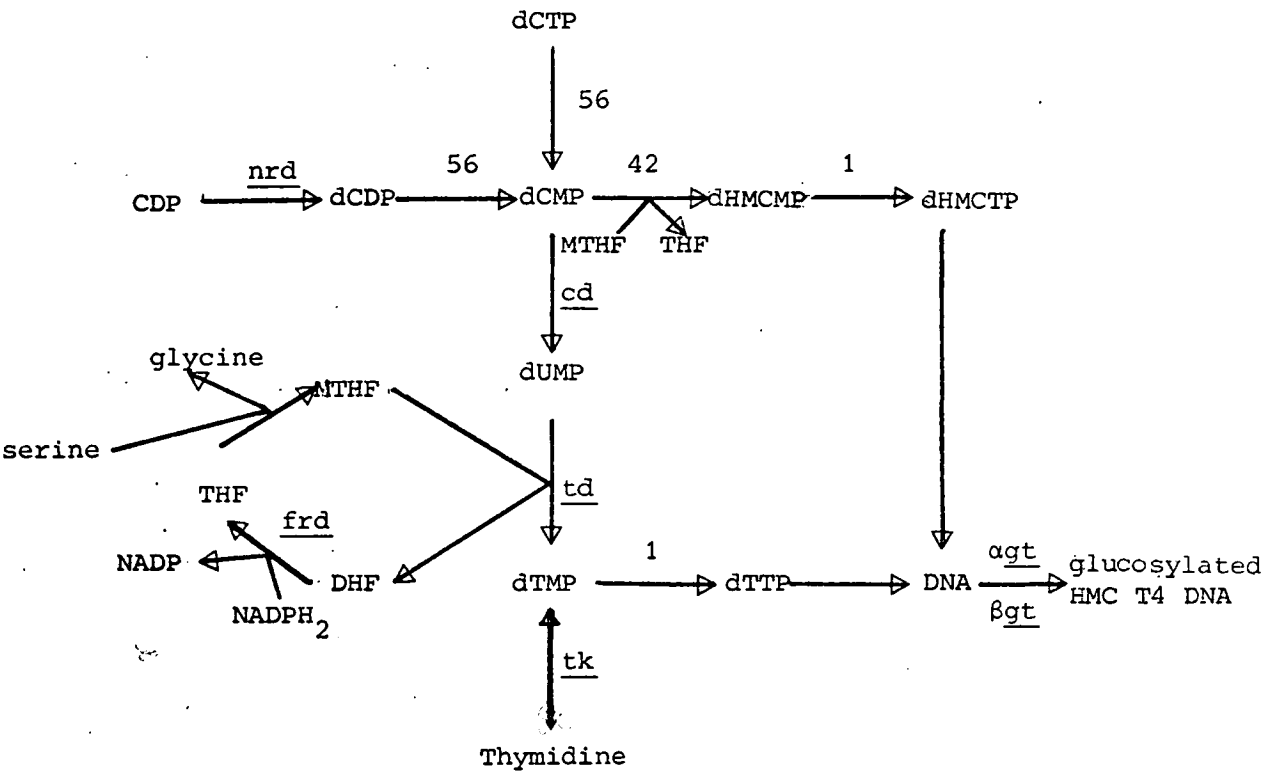
Nucleotide metabolism in T4 infected cells. The T4 genes whose products are involved in these processes are indicated beside the arrows.

MTHF = methyltetrahydrofolate

THF = tetrahydrofolate

DHF = dihydrofolate

Figure 1.4


dCTP
56
CDP
nrd
dCDP
56
dCMP
42
dHMCMP
1
dHMCTP
MTHF
THF
cd
dUMP
glycine
MTHF
serine
td
THF
frd
NADP
DHF
NADPH2
dTMP
1
dTTP
DNA
αgt
βgt
glucosylated
HMC T4 DNA
tk
Thymidine

e.g. frd, td, nrdA and nrdB (Hall et al, 1967), and common control: for instance, mutants selected to overproduce gp frd also overproduce gps cd, tk and 56 and underproduce gp td (Johnson and Hall, 1974).

(e) T4 transcription

T4 transcription is achieved by the host RNA polymerase, although T4 encoded functions alter and bind to the polymerase during infection. Transcription can be broadly separated into two phases, pre-replicative and post-replicative, since a dramatic change is seen in the polypeptide band pattern on SDS polyacrylamide gels of T4 infected cells after the onset of DNA replication (Wiberg et al, 1962). Three classes of T4 genes are defined; early genes, whose products only appear before replication; quasi-late genes, whose products appear before replication but increase in abundance after replication; and late genes whose products only appear after replication (O'Farrell and Gold, 1973).

Early T4 gene products show characteristic times of appearance, which implies that control mechanisms exist within early gene expression. All known early genes are transcribed from the l-strands of T4 DNA and the majority of late genes from the r-strand (Guha et al, 1971).

(i) Early transcription

During the first minutes of infection, early T4 mRNA and tRNA synthesis is concurrent with rapidly declining host RNA synthesis (Kennel, 1970). Early proteins appear in their characteristic order during the first five to six minutes of infection and no new proteins appear after this time until the onset of DNA replication. Early mRNA is not produced by a single coordinate transcriptional unit, although early transcriptional

units do exist. 99% of early mRNA is read off the l-strand and this falls to about 95% just after the start of DNA replication (Guha et al, 1971).

Early genes code for functions involved in phage specific nucleotide and nucleic acid metabolism, binding cell membranes and the alteration of the host-transcriptional and translational machinery, and include both essential and non-essential genes. Early genes are clustered in two regions of the genome at 120-147 kb and 158-75 kb. (Wood and Revel, 1976; see Figure 1.2).

Transcription and translation are coupled so that specific mRNAs and their products are detectable almost simultaneously (Trimble et al, 1972). An exception to this coupling is T4 lysozyme, the product of the e gene, whose transcription starts during the first minutes of infection, but whose product does not appear until after DNA replication (Jayaraman and Goldberg, 1970).

T4 RNA chains grow about three times slower than those of E.coli or phage T7 (see Rabussay and Geiduschek, 1977a). This does not involve a postinfection decrease in ribonucleotide pool size (Mathews, 1972), although it could be the result of T4 DNA modification, such as the presence of glucosylated HMC (Cox and Conway, 1973). Translation of T4 mRNA is comparably slower (Gausing, 1972) and as early T4 mRNA is relatively stable (Sauerbier et al, 1969) slow transcription could be a result of slow translation.

(ii) Early transcriptional classes

More than one class of early mRNA exists by several criteria: the appearance of a large class of early mRNA is blocked by the addition of chloramphenicol at the time of infection, although this effect disappears within two to three minutes at 30°C (Salser et al, 1970);

this effect of chloramphenicol seems to be transcriptional and not translational as early transcription is insensitive to the presence of amino acid analogues or miscoding inducing antibiotics (Black and Gold, 1971; Brody, 1975); some early mRNA, e.g. that of g 32, is delayed in initiation until one to two minutes after infection as their presence is sensitive to rifampicin before this time (O'Farrell and Gold, 1973); the yield of some early gene products, e.g. gp 43, is dependent on this delayed mRNA initiation event (Hercules and Sauerbier, 1974); the synthesis of gene products dependent on this delayed initiation event, occurs in *in vitro* systems derived from uninfected *E.coli* cells, and *in vivo* generated mRNA, but not in a coupled transcription, translation system primed with T4 DNA (Gold *et al*, 1973); "antimessenger RNA", a set of early transcripts that are complementary to a fraction of late mRNA, whose function is unknown, and which first appears two to three minutes after infection, is dependent on protein synthesis, seems to be produced by the relief of attenuation, although the existance of leader sequences has never been demonstrated in T4 infected cells (Notani, 1973; Rabussay and Geiduschek, 1977a); mutations in the *mot* gene specifically affect the production of proteins that are in any way dependent on the delayed initiation event and the production of antimessenger (Mattson *et al*, 1974; Snyder, 1975).

The effect of chloramphenicol on transcription seems to be mainly an induced polarity (Young, 1975), which is also seen in bacterial operons, e.g. the *trp* operon (Morse and Yanofsky, 1969). The polarity induced by chloramphenicol in the *trp* operon varies when transcription is initiated at different promoters (Imamoto, 1973). Chloramphenicol induced polarity in T4 varies with time of addition

after infection and between messages (Brody et al, 1970), which implies that the relief of chloramphenicol induced polarity, two to three minutes after infection, depends on a change in transcriptional specificity involving new promotors.

Early genes are subdivided into immediate early (IE) or delayed early (DE) according to the time of appearance of their products. This difference in appearance time partly reflects promotor proximity. IE genes are proximal to early promotors (P_E) which are recognised by the host RNA polymerase immediately after infection and DE genes are distal to the same promotors. This means that transcription and translation of IE genes occurs before that of DE genes (Milanesi et al, 1970; Brody et al, 1970; Salser et al, 1970; O'Farrell and Gold, 1973; Hercules and Sauerbier, 1973). A typical T4 early transcriptional unit consists of a P_E promotor, an IE gene and one or more DE genes, with at least one site between the IE and DE genes that is sensitive to chloramphenicol induced polarity. This same region must also contain sites sensitive to ρ-induced RNA chain termination at low ionic strength in vitro, as only IE genes are transcribed by the host RNA polymerase under these conditions in the presence of ρ (Richardson, 1970; Jayaraman, 1972). An example of such a transcriptional unit is that containing the DE genes rIIA and rIIB, which are found on a polycistronic message early in T4 infection, initiated upstream of rIIA (Bautz et al, 1969).

T4 DNA also possesses intrinsic in vitro transcription termination signals that cause the release of RNA polymerase and RNA ending in 3'U from the DNA template (Millette et al, 1970) Such RNA chains are 7-12,000 nucleotides long and as T4 transcription progresses at 1100 nucleotides/minute at 30°C (Bremer and Yuan, 1968)

and no early protein makes its first appearance after 6 minutes of infection (O'Farrell and Gold, 1973), these RNA chains must contain more than one early transcription unit (Brody and Geiduschek, 1970). Transcripts of this length are not seen *in vivo* indicating that either accessary RNA chain terminating factors exist *in vivo* or that T4 RNA is quickly processed after production.

The most straightforward explanation of the early transcriptional regulatory event that occurs one to two minutes into infection, is that transcription starts to be initiated from a different set of promotors. These have been termed middle promoters (P_M) by Rabussay and Geiduschek (1977a) and are the same as the quasi-late promotors (P_Q) of O'Farrell and Gold (1973). The former term is preferable as the term quasi-late has been used to describe different regulatory groups. P_M promotors can be located within P_E initiated transcription units, thus some early genes can be read from different promotors, explaining why the *r*IIB transcript can be found both to the 3' side of the *r*IIA transcript on a polycistronic early mRNA and at the 5' end of another early message that lacks the *r*IIA transcript (Schmidt *et al*, 1970). It seems that a P_M promotor is located within the *r*IIA gene and this can initiate *r*IIB transcription (Singer *et al*, 1976). mRNA of g 1 can be found in both mono- and poly-cistronic forms which must reflect different transcriptional starts or RNA processing (Sakiyama and Buchanan, 1972). Only a few early genes seem to be transcribed from P_M promotors and these include g 1 (Gold *et al*, 1973).

IE genes can be transcribed from P_M as well as P_E promotors and typically these are genes whose products are required throughout infection such as genes *ip*I, *ip*II and *ip*III that code for the phage

internal proteins, implicated in DNA packaging (Brody et al, 1971).

Ultra-violet (UV) irradiation is thought to inhibit transcription, by impeding the progress of RNA polymerase at induced pyrimidine dimers (Sauerbier et al, 1970). Thus the UV sensitivity of the synthesis of a particular gene product, is proportional to the distance of the structural gene involved from the promotor that initiates its transcription. The UV sensitivity of the appearance of gps 43 and 45 alters relative to that of gp αgt during infection significantly decreasing a few minutes into infection (Hercules and Sauerbier, 1974). This strongly indicates that g 43 and g 45 are transcribed from different promotors a few minutes into infection.

Transcriptional control may also be entirely or partly due to an altered termination specificity. Such a process would require a regulatory substance with antitermination activity, possibly analogous to the λN gene product (Franklin, 1971). Such termination sites cannot be the same as the intrinsic RNA termination sites as they are not recognised in vitro. This type of transcriptional control cannot solely account for the appearance of early mRNA species carrying the rIIB transcript at its 5' end (see above).

The analysis of the early regulatory event should improve due to the recent isolation of mutants defective in the process; mot and farI mutants (Mattson et al, 1974); farI mutants were isolated as mutants resistant to folate analogues (Chace and Hall, 1975a) and seem to be allelic to mot. Most mot mutations delay but to not abolish the early regulatory event, but a conditionally lethal mot mutant has now been isolated (Mattson et al, 1979). farI mutants delay the early regulatory event, but also overproduce

the rIIB product. The effector of the early regulatory event is diffusible (Daegelen, 1975), thus the product of the mot-farI gene(s), whose product is also diffusible, is a candidate for such an effector. A recently devised in vitro system that reproduces the induced polarity effect of chloramphenicol and certain aspects of the mot phenotype, indicates that the early regulatory event involves a modification of the DNA template, perhaps a non-covalent cell membrane interaction (Thermes et al, 1976; Daegelen et al, 1975). The early regulatory event occurs in the presence of amino acid analogues and antibiotics that induce miscoding, thus it is likely that the effector of the event is preformed. If this preformed effector is derived from the host, perhaps it involves a membrane component, as the early regulatory event seems to involve membrane association.

T4 promotors should become better understood in the near future, as the application of molecular cloning to the T4 system, facilitates the DNA sequencing of T4 promotors, and sophisticated analyses of the regulatory process.

(iii) RNA polymerase in early transcription

Host RNA polymerase is used throughout T4 infection, in fact rifampicin inhibits all types of T4 transcription (Haselkorn et al, 1969), and the presence of all polymerase subunits can be demonstrated at all times during infection (Goff and Weber, 1970). However host polymerase is subject to chemical alterations and binds specific T4 proteins during infection.

The product of the T4 alt gene is injected into the host during infection (Rohrer et al, 1975) and probably catalyses the addition of an ADP-ribose molecule to one of the two α subunits of the

polymerase, although a fraction of σ, β and β' subunits become phosphorylated at the same time (Seifert et al, 1971; Roherer et al, 1975). This process, termed alteration, occurs in the presence of chloramphenicol, because the alt protein is preformed (Seifert et al, 1969). Alteration is reversible and transient in vivo and could be involved in the shut off of host transcription since a mutation in a gene coding for an RNA polymerase subunit has been reported that leads to inefficient host transcriptional shut off and seems to be defective in alteration (Snyder, 1973).

A second event, modification, is catalysed by the phage mod gene product and involves the ADP-ribosylation of all polymerase α subunits (Goff, 1974). This addition seems to involve a different site on the α subunit from that involved in alteration. Here the ribose carbon-1 atom, binds to the guanido nitrogen atom of an arginine residue on the α subunit. Modification is sensitive to chloramphenicol and thus requires protein synthesis (Seifert et al, 1969). This process also seems to contribute to host transcriptional shut off, since modified polymerase is inefficient in the transcription of E.coli DNA in vitro, but efficient with T4 DNA (Mailhammer et al, 1975). Neither modification nor alteration is essential for T4 development.

T4 infected cells have been reported to contain an activity that stimulates the transcription of DE genes, with the core RNA polymerase from RNA infected or uninfected E.coli cells (Travers, 1970). This activity which should interact with P_M promotors has different fractionation properties from the host σ factor and could either be an initiation factor used instead of σ, or a complex of σ and another protein (Losick, 1972). It is possible to isolate several

non-adsorbed proteins during the purification of altered RNA polymerase, which inhibit both core and holoenzyme of the host and the altered core enzyme on either calf thymus or T4 DNA templates (Rabussay, 1972; quoted by Rabussay and Geidnschek, 1977a). The interaction of host σ factor with inhibitory factors provides another means by which transcriptional specificity could be altered.

(iv) Shut off of early protein synthesis

Some early genes, e.g. tRNA genes, are expressed throughout infection, but others are shut off after about 12-14 minutes at 30°C (Hosoda and Levinthal, 1968). This shut off is asynchronous, presumably reflecting diverse regulatory phenomena controlling the process and is probably a result of both transcriptional regulation and an alteration in the stability and activity of early mRNA (Salser et al, 1970; Bolle et al, 1968). Prior to shut off, functional T4 mRNA is stable with a half life of 8-10 minutes at 30°C, measured on addition of rifampicin 1 minute after infection (Craig et al, 1972), but after shut off of early protein synthesis, early mRNA is rapidly degraded, half lives of functional mRNA here being 2-3 minutes at 37°C (Sauerbier and Hercules, 1973). This change in functional half life could either be direct, as a result of a chemical change in mRNA, or indirect, due to message competition for the translational machinery. If early mRNA was competed out by late mRNA, it would be rapidly degraded as the uncoupling of transcription and translation of E.coli mRNA decreases its stability (Hansen et al, 1973). Such mRNA competition could be very effective as T4 infected cells are at messanger excess, translation not being limited by mRNA concentration (Cohen et al, 1972), but is not exaggerated by late mRNA specific translation factors

because late proteins are produced in *in vitro* systems derived from uninfected *E.coli* cells and late T4 mRNA (Wilhelm and Haselkorn, 1971).

g1 expression is subject to post-transcriptional control. Although the appearance of its product is blocked in the absence of DNA replication, its functional mRNA remains in the cell while general protein synthesis continues (Cohen, 1972; Sakiama and Buchanan, 1972). The shut off of synthesis of other early gene products still occurs in the normal sequence in the absence of DNA replication, but is much delayed (e.g. Wiberg *et al*, 1973). The decay of g1 mRNA activity depends upon replication and not late protein synthesis (Sakiama and Buchanan, 1972), but the total amount of gp 1 in an infected cell depends on late protein synthesis and not replication (Bolund, 1973).

The *regA* gene of T4 has an effect on early mRNA stability. Early mRNA stability is greatly increased in a *regA* replication defective background than in a wild type or *regA* background (Wiberg *et al*, 1973).

The shut off of early protein synthesis is a complex process and is not well understood at present.

(f) Translational elements

T4 codes for various components either used in translation or that modify existing host translational components. None of these functions is essential for T4 development but they can be conditionally lethal on certain bacterial strains (Guthrie and McClain, 1973). These include: a function that modifies existing tRNAs, for example the specific cleavage of host leucyl-tRNA (Yudeleuich, 1971); functions that influence host tRNA modifying enzymes (Boezi *et al*, 1967); a function that alters valyl-tRNA synthetase (Muller and Marchin, 1975);

functions that alter translation inhibitory factors (Wahba, cited by Rabussay and Geiduschek, 1977a); and eight phage encoded tRNA species that can be charged with arginine, glycine, isoleucine, leucine, proline, serine, glutamine and threonine (McClain et al, 1972).

These functions probably ensure that the host's translational machinery is biased towards the translation of T4 mRNA and to T4 message codon usage. Recent evidence shows that ribosomes isolated at late times into T4 infection translate mRNA 40-50% more slowly than those isolated at early times (Pennica and Cohen, 1978). The function of this change is unknown.

(g) Autoregulation

Amber mutants of genes 32 and 43 overproduce their respective truncated products, indicating that their expression is negatively controlled by their products (Russel, 1973; Russel et al, 1976). gp 43 synthesis is also dependent on DNA replication and is over-produced in the absence of replication. Thus g 43 seems to share some regulatory features with other late early genes, and indeed its expression is shut off at about the same time as other early proteins (Russel, 1973).

Autoregulation is the main method of control of g 32 expression as g 32 amber mutants continually synthesise their truncated polypeptides throughout infection (Russel et al, 1976). gp 32 binds to single stranded DNA and its synthesis is derepressed by single stranded DNA. Thus gp 32 synthesis is coupled to cellular processes that expose single stranded DNA during infection (e.g. Broker and Lehman, 1971; Yeh and Wu, 1973). These are the processes of DNA recombination, replication and repair that are known to require gp 32. gp 32 remains active after release from DNA and as single stranded

DNA is present only transiently in infection, synthesis of this protein is shut off once a constant pool of replicating DNA has been achieved (Krisch et al, 1974).

gp 32 is autoregulated at the post-transcriptional level. gp 32 is thought to bind to its mRNA in such a way as to abolish its transcriptional activity while not affecting the activity of other mRNA species (Russel et al, 1976).

(h) DNA replication

T4 DNA becomes membrane associated early in infection, a process that is independent of T4 gene expression (Miller, 1972; Earhart et al, 1973). Replication seems to occur preferentially around sites of membrane attachment and some mutations lending to the arrest of DNA replication also involve the disruption of this membrane attachment (Huberman, 1968; Wu and Yeh, 1974).

T4 DNA replication starts about 6 minutes after infection and proceeds bidirectionally from multiple origins (Carlson, 1973; Howe et al, 1973; Kozinski and Doermann, 1975). However, this was difficult to prove using standard techniques due to the complex nature of replicating T4 DNA, the high recombination frequency, the asynchrony of replication rounds and the circular permutation of the genome. A very recent study using cloned T4 DNA fragments has revaled the presence of a major origin of replication in the g 50-g 5 region and a minor origin in the g w-g 29 region (Halpern et al, 1979). This method should facilitate the locating of other origins.

Replication and recombination produce the large concatemeric lengths of T4 DNA necessary for packaging (Miller et al, 1970; Broker, 1973). In fact all replicating DNA in a T4 infected cell seems to be linked into a complex network by recombination events (Huberman, 1968).

Replication is carried out by the T4 encoded DNA polymerase, gp 43 (Dewaard et al, 1965), although many other T4 products participate in the process. It seems that many T4 gene products associate into a replication complex that includes gps 43, 32, 44, 62, 45, 42 and 1, although such a complex cannot be tightly associated as it has never been isolated. However binding between gps 43 and 32 can be demonstrated (Huberman et al, 1971); gps 44 and 62 form a stable complex (Barry and Alberts, 1972); a complex of gps 44, 62 and 45 has a DNA dependent ATPase activity in which the DNA binding site resides on the gp 44/62 complex (Piperno et al, 1978); gps 44, 45 and 62 interact with gp 43 (Alberts et al, 1973, 1975); gps 42 and 1 also seem to have structural replication roles (Wovcha et al, 1973; Chiu et al, 1976). An in vitro system involving gps 43, 32, 41, 44, 62 and 45 is capable of synthesising large pieces of T4 DNA at approximately the in vivo rate, but this does not exclude the involvement of other proteins (Alberts et al, 1975).

(i) Late transcription

Two main types of mRNA are produced by late transcription, that is transcription after the onset of replication: that which is produced at low levels pre-replicatively and at higher levels post-replicatively (quasi-late mRNA), and that which is only produced after replication starts (true-late mRNA). The synthesis of these two forms of late mRNA differs, in both RNA polymerase and DNA template requirement and the type of regulatory mechanisms that govern their synthesis.

True-late transcription and translation, produce a set of proteins that appear almost simultaneously a few minutes after replication begins; that is about 9 minutes into infection (Hosoda and

Levinthal, 1968; Notani, 1973). True-late proteins include the phage coat proteins and those required for phage assembly. The mRNA of true-late genes is mainly transcribed from the r-strand (Guha et al, 1971), but the synthesis of l-strand true-late mRNA is under the same control as that of r-strand mRNA (Notani, 1973).

Quasi-late gene products are typically those that are required continuously throughout infection and include gp 32 (Krisch et al, 1974) and the phage internal proteins (O'Farrell and Gold, 1974). It is expected that quasi-late genes will have the same polarity as the early genes and so be read off the l-strand because the vast majority of pre-replicative mRNA has this polarity (Notani, 1973; Guha et al, 1971). This has been shown to be the case for gene e (Jayaraman and Goldberg, 1970). Quasi-late expression seems to be regulated in a non-uniform manner (Guha et al, 1971).

True-late gene expression requires modifications to both RNA polymerase and DNA template. The DNA template can only be competent for true-late transcription if it contains HMC instead of cytosine and is in a 'processed' state, the nature of which is not understood (Kutter et al, 1975; Wu and Geiduschek, 1974).

True-late transcription beings about 9 minutes into infection and about 3 minutes after the onset of DNA replication (Rabussay and Geiduschek, 1977a). The time difference between the starts of these two linked processes may either reflect the time necessary for the accumulation and assembly of components needed for true-late transcription, or the incompetence of DNA in early rounds of replication to sponsor true-late transcription. In fact it seems that the mechanism of replication alters after the first few rounds (Broker and Doermann, 1975).

The HMC requirement of true-late transcription is not total, as up to 20% of the HMC residues of T4 DNA can be replaced by cytosine without affecting true-late transcription (Kutter and Wiberg, 1969). Mutants of genotype g 42^-, g 56^-, denB$^-$ can produce T4 DNA in which all HMC residues are replaced by cytosine, but are blocked in late transcription (Wilson et al, 1977; Morton et al, 1978). This block can be relieved by the presence of a mutation in the alc gene (Snyder et al, 1976). It seems that the alc gene product is responsible for the HMC specificity of true-late transcription. Quasi-late gene expression is not abolished in g 56^-, denB mutants as gp 32 is overproduced (Kutter et al, 1975).

β-glucosylation affects true-late transcription, but cannot be essential as T4 can develop normally in the absence of glucosylation (Revel, 1967). Some host RNA polymerase mutants render both true-late transcription and initial DNA replication cold sensitive and impair host shut off functions. The transcription and replication cold sensitivities can be compensated by either the absence of β-glucosylation (α-glycosylation has no effect), certain g 45 mutations that lead to the underproduction of gp 45 or an uncharacterised mutation, gor 2, that maps between genes 55 and αgt (Snyder and Montgomery, 1974).

True-late transcription seems to require a modification to normal linear duplex DNA. Effective transcription here is linked to DNA replication (Riva et al, 1970) and the greatly reduced level of true-late transcription that occurs in the absence of replication, can be increased by mutations affecting DNA structure (Wu et al, 1975; Hosoda and Levinthal, 1968; Bolle et al, 1968). True-late transcription also depends upon continued replication since the

shifting of a g 43 temperature sensitive mutant to the non-permissive temperature, reduces the level of transcription by ten-fold in a few minutes (Riva et al, 1970). The low level of true-late transcription seen in the absence of replication could either reflect another mode of transcription normally masked by replication-linked transcription, or that the replication requirement of true-late transcription is not absolute, but stimulatory.

The types of mutation that give enhanced true-late transcription in the absence of replication, mentioned above, are those that give rise to the formation of stable interruptions in the primary or secondary structure of the DNA template and include mutations in genes 30 (DNA ligase), 41 (mutations in which produce single stranded regions) and 46 and 47 (exonuclease subunits). In fact combinations of g 30 and g 46 mutations give good levels of true-late transcription (Wu et al, 1975). This suggests that true-late transcription requires the presence of nicks or single strand gaps or the DNA secondary structure disruptions caused by such primary leisions (Riva et al, 1970). However the distribution of breaks is not significantly different between the l- and r-strands in both normal and replication deficient phage (Wu et al, 1975), and no known gene product contributing to true-late transcription has an endonuclease activity.

gp 45 is essential both as a member of the DNA polymerase complex and in true-late transcription. gp 45 binds RNA polymerase, suggesting that true-late transcription could be initiated at replication forks where it would provide a link between transcription and replication (Ratner, 1974). If true-late transcription is initiated at single strand gaps on replication forks, the mutations

leading to replication independent true-late transcription, could mimic such gaps. Perhaps either true-late promotors cannot be recognised in intact duplex DNA or such DNA causes the premature termination of true-late transcripts. DNA replication *in vitro* is greatly stimulated by the presence of the 4 ribonucleotide triphosphates in the absence of RNA polymerase (Alberts *et al*, 1975). This could indicate that if this phenomenon reflects a mechanism for RNA primer synthesis that does not involve RNA polymerase, true-late transcription may be initiated in an unconventional way.

The product of the *pseT* gene has recently been shown to affect true-late transcription (Sirotkin *et al*, 1978). *pseT* is a non-essential gene, but *pseT*$^-$ mutants grow very poorly in Lit$^-$ bacterial hosts due to defective true-late transcription (Cooley *et al*, 1979). It seems that gp *pseT* may be involved in the processing mechanism that renders T4 DNA competent for true-late transcription.

(j) RNA polymerase modifications in true-late transcription

True-late transcription is achieved by a modified host RNA polymerase that has 95% of its α subunits ADP ribosylated (α_m) and a proportion of its β, β' and α subunits altered (Goff, 1974; Rohrer *et al*, 1975) and is directly influenced by gps 33, 45 and 55, with gps 45 and 55 being required continusously (Wu *et al*, 1975; Coppo *et al*, 1975a, 1975b). gp 45 is required for both replication and true-late transcription since it is essential for replication independent true-late transcription and some mutations in g 45 can suppress some host mutations (*tabD*) that affect true-late transcription and map in the structural gene for the RNA polymerase β subunit (Wu *et al*, 1975; Coppo *et al*, 1975a). This genetic

evidence of a gp 45, RNA polymerase interaction, is supported by the biochemical evidence that gp 45 is retained significantly on a sepharose-RNA polymerase affinity column, but only if the polymerase involved is the T4 modified core (Ratner, 1974). This indicates a functional difference between modified and unmodified polymerase, which cannot be essential as T4 development is normal in its absence (Horwitz, 1974). However a tightening of an existing weak binding between gp 45 and the unmodified polymerase by modification would benefit true-late transcription.

gp 55 binds tightly to RNA polymerase and certain mutations in this gene suppress tabD host mutations that map in the structural gene for the β' subunit of RNA polymerase (Coppo et al, 1975a). gp 55 remains bound to RNA polymerase through all normal polymerase purifucation steps, but is present in substoichiometric amounts (Horvitz 1973, 1974; Goff and Weber, 1970).

gp 33 also binds to polymerase and copurifies with it, although separation is possible by phosphocellulose chromatography (Horvitz, 1973). gp 33 seems to compete with σ factor for the same binding site on the core polymerase, but does not function as a σ analogue, as late polymerase core is only as active as the host core in transcribing, T4 or calf thymus DNA (Ratner, 1974; Schachner et al, 1971). A recently devised in vitro transcription system has shown that the T4 modified or host core transcribe late T4 genes in the absence of σ (Rabussay and Geiduschek, 1977b). The presence of σ prevents the polymerase core from binding DNA nonspecifically and RNA chain initiation at nicks that seem necessary for true-late transcription (Chamberlin, 1974). Thus gp 33 may act in displacing σ from polymerase to enable true-late transcription to occur.

Two phage specified early proteins also bind to the late polymerase and have sizes of apparent Mr 15,000 and 10,000 (Stevens, 1972). The Mr 15,000 protein is the product of the alc gene (Sirotkin et al, 1977) and probably specifies the HMC requirement of true-late transcription. The nature and role of the Mr 10,000 protein are unknown.

A model for late transcription has been proposed (Rabussay and Geiduschek, 1977a) and involves quasi-late transcription being effected by a polymerase of structure β, β', $(\alpha_2)_m$, 15 K, 10 K probably requiring host σ factor for initiation, and true-late transcription being effected by a polymerase of structure β, β', $(\alpha_2)_m$, 15 K, 10 K, gp 33, gp 45, gp 55 without the requirement of host σ. Since RNA polymerase core can initiate RNA chains at nicks, an activity inhibited by σ (Chamberlin, 1974), and nicks are implicated in true-late transcription, σ has the potential of positively regulating quasi-late transcription and negatively regulating true-late transcription. If this happens, the relative amounts of quasi-late and true-late transcription will depend on the relative concentrations of σ and gp 33.

(k) Packaging and assembly

DNA packaging occurs by a headful mechanism off concatemeric DNA molecules (Ritchie and White, 1972). These concatemers are produced by recombination and not rolling circles (Frankel, 1968). The headful packaging method does not involve sequence specific endonucleotytic cuts as daughter DNA molecules are circularly permutted. A DNA molecule 2% longer than one whole genome is packaged (MacHattie et al, 1967), thus an entire copy of the genome is included into the phage particle. This packaging method explains

both the mechanism of generation of terminal redundancy and the circularity of the genetic map, as subsequent chromosomes cut off the same concatemer will have different starts.

gp 49 is essential for packaging and temperature sensitive mutants here have the property of producing empty heads at the restrictive temperature and full heads at the permissive temperature (Laemmli et al, 1974). The action of gp 49 is unknown.

The assembly of the complex phage coat structure depends upon many gene products and occurs in a rigid sequence of events (see Casjens and King, 1975). Head formation involves the participation of at least 18 T4 gene products including gp 23, the major head structural protein; tail formation requires at least 21 gene products, most of which have structural roles, and in addition several non-essential structural components such as gp td, gp frd and the unusual folate form dihydropteryl hexaglutamate (Kozloff et al, 1975a, 1975b, 1975c); and tail fibre formation requires at least 7 gene products 2 of which act catalytically. These 3 events occur independentaly and simultaneously. Completed heads then attach to completed tails (Edgar and Lielausis, 1968) and completed tail fibres attach to this complex, a process catalysed by gp 63 (Wood and Henninger, 1969), to produce complete, infective phage particles.

1) Lysis

T4 infected cells have long been known to produce a lysozyme function that digests the peptidoglycan of the host cell wall (Streisinger et al, 1961). T2 carries this lysozyme as a tail base plate component to assist the infection process (Koch and Dreyer, 1958) but the T4 lysozyme has no such structural role,

rather gp 5 provides the infective lytic function (Kao and McClain, 1980). Such functions explain the 'lysis from without' phenomenon first described by Delbruck in 1940.

T4 lysozyme is the product of the e gene and its expression is anomalous (see sections e) (i) and i)). Lysozyme accumulates from just after the onset of replication but cannot function unless it gains access to the bacterial cell wall. This access is provided by the T4 gt product which causes membrane damage and uncouples oxidative phosphorylation and electron transport within the membrane (Josslin, 1971). Cell lysis normally occurs about 30 minutes after infection and liberates about 100 progeny phage into the medium (Ellis and Delbruck, 1939).

Lysis is complicated by the phenomenon of lysis inhibition (Doermann, 1948). This occurs when a T4 rII$^+$ phage superinfects a T4 rII$^+$ infected cell. It seems that the action of lysozyme is inhibited by the superinfecting phage so that lysis is delayed by a few hours. When lysis finally occurs, about 1000 progeny phage are released. The inhibition of lysis seems to be indirect, with the primary effect being a change in the cell membrane that renders it insensitive to the action of gp t (Josslin, 1971).

The g 30-g 32 Region of the T4 Genome

This region of the T4 genome stretches from 122 to 145 kb on the T4 map (see Figure 1.2) and includes 13 genes which are either under early or quasi-late control (Wood and Revel, 1976). Only four genes, genes 30, 31, 32 and 63 are essential for T4 development in normal laboratory strains. The genes in this region and the functions of their products are listed below.

(a) g 32

gp 32, Mr 36,000 (O'Farrell et al, 1973) is a DNA binding protein and an essential component of both DNA replication and recombination processes (Epstein et al, 1963; Tomizawa et al, 1966). Binding is preferentially to single stranded DNA (Alberts et al, 1968). Each molecule binds to about 5 nucleotides and this binding is cooperative, facilitating saturation of single stranded regions at only moderate gp 32 concentrations (Jensen et al, 1976; Alberts et al, 1968; McGhee and von Hippel, 1974). Protein-protein interactions, involving overlaps between adjacent molecules, are implicated in this cooperativity (Alberts, 1970). The N-terminal portion of gp 32 is involved in DNA binding whereas c-termination portion is involved in DNA replication and recombination (Mosig et al, 1977). gp 32 is required throughout infection, is subject to quasi-late control (Alberts and Frey, 1970) and seems to function by stabilising single stranded DNA intermediates in replication and recombination. gp 32 represses its own synthesis at the translational level (Russel et al, 1976).

(b) frd

gp frd Mr 22,ooo (Mosher and Mathews, 1979) is the phage encoded dihydrofolate reductase, which catalyses the production of tetrahydrofolate from dihydrofolate in the presence of NADPH (Hall, 1967). It is non-essential for T4 development but ensures that thymidine concentrations in the infected cell are high after the shut off of host functions, by its involvement in recycling the methylene tetrahydrofolate necessary for the thymidylate synthetase reaction. Dihydrofolate reductase is the target enzyme of folate analogue drugs such as trimethoprim, thus, by increasing the level

of this enzyme within the cell, T4 can grow in E.coli at concentrations of trimethoprim that are inhibitory to uninfected cells (Chace and Hall, 1975a). gp frd is also a non-essential component of the phage base plate (Kozloff et al, 1975a) and is subject to either early or quasi-late control (Wood and Revel, 1976).

(c) td

gp td, Mr 29,000 (Capco et al, 1973) is the phage encoded thymidylate synthetase, which catalyses the production of dTMP, water and dihydrofolate from methylene tetrahydrofolate and dUMP (Shapiro et al, 1965). gp td is non-essential for T4 development and td mutants can grow on E.coli thymidylate synthetase mutants (thyA), although with a reduced burst size, as thymine required for phage DNA replication can be scavenged from the breakdown products of host DNA (Simon and Tessman, 1963). However, gp td is thought to contribute to the maintenance of optimal intracellular thymidine concentrations after host shut off. gp td is also a non-essential component of the phage base plate (Kozloff et al, 1975b) and is subject to either early or quasi-late control (Wood and Revel, 1976).

(d) nrdA and nrdB

gps nrdA and nrdB, Mr 80-85,000 and 35,000 respectively (Berglund, 1975) are the α and β subunits of the phage encoded ribonucleotide diphosphate reductase, which catalyses the formation of deoxyribonucleotide diphosphates from ribonucleotide diphosphates with the participation of the T4 thioredoxin, the product of the unlinked nrdC gene (Yeh and Tessman, 1972; Tessman and Greenberg, 1972). These gene products are non-essential for T4 development, but again help to ensure good concentrations of precursors for DNA synthesis after host shut off. nrdA and B are under early or quasi-late control (Wood and Revel, 1976).

(e) denA

gp denA is the phage encoded endonuclease II, which catalyses the formation of nicks at the 5' side of cytosine residues in duplex DNA (Sadowski and Hurwitz, 1969). This is the main endonuclease responsible for the breakdown of host DNA (Warner et al, 1970). denA is subject to early or quasi-late control (Wood and Revel, 1976). The size of the denA product is unknown.

(f) g 63

gp 63, Mr 42,000 (Vanderslice and Yegian, 1974) was originally identified as a function catalysing tail fibre attachment to the base plate (Wood and Henninger, 1969) and is now known to also be the phage encoded RNA ligase (Snopek et al, 1977). RNA ligase catalyses the formation of phosphodiester bonds between 3'-OH and 5'-phosphate residues on ribonucleotides and requires the presence of ATP and magnesium ions (Silber et al, 1972). It has been claimed that RNA ligase has a stimulatory effect on the blunt end ligation activity of T4 DNA ligase (Sugino et al, 1977), but the production of pure DNA ligase has shown that if RNA ligase has such a role, it is not essential (Murray et al, 1979).

The role of RNA ligase in vivo is unknown, but it could act in RNA processing. γ^{32}P ATP incorporation into polyribonucleotides, using a system derived from T4 infected cells, is mainly at the 5' end, implying that this addition is catalysed by the T4 polynucleotide kinase. However some γ^{32}P is present within phosphodiester bonds, except if the T4 strain used carries a g 63 mutation, suggesting that the presence of label of this nature is due to successive kinase and RNA ligase reactions (David et al, 1979). Again this suggests that RNA ligase plays a role in RNA processing, g 63 is subject to quasi-late control (Wood and Revel, 1976).

(g) alc

This gene was first identified as a function involved in the unfolding of the host chromosome, unf (Snustad et al, 1976) and was later found to be allelic with a function involved in binding late RNA polymerase and providing the requirement of late transcription for an HMC containing template (Sirotkin et al, 1977). alc mutations were first identified as those that allowed true-late gene expression from T4 DNA templates which contained cytosine in place of HMC (Snyder et al, 1976). gp alc also seems to participate in the shut off of host transcription as alc mutants allow the synthesis of some host RNA after infection (Sirotkin et al, 1977). Since RNA is involved in the maintenance of the host chromosome structure (Pettijohn and Hecht, 1973) and some types of transcription possibly depend on chromosome structure, it seems likely that the alc and unf phenotypes are causally related; gp alc either shuts off RNA synthesis causing host chromosome unfolding, or unfolds the host chromosome leading to the shut off of RNA synthesis. gp alc also causes the shut off of late transcription (Pearson and Snyder, 1980) and is the Mr 15,000 protein that binds to late RNA polymerase (Sirotkin et al, 1977). alc is subject to early or quasi-late control (Wood and Revel, 1976).

(h) pseT

This gene product was first identified as the 3' phosphatase induced in T4 infected cells (Depew and Cozzarelli, 1974) and has now been shown to also be the phage encoded polynucleotide kinase (Sirotkin et al, 1978). The 3' phosphatase activity catalyses the dephosphorylation of 3' phosphoryl groups on DNA molecules and the polynucleotide kinase activity transfers the γ phosphate of nucleotide

triphosphates (usually ATP) to the 5'-OH terminus of a polynucleotide. While the former reaction is very specific, the 3' phosphoryl groups of RNA molecules not being substrates (Depew and Cozzarelli, 1974), the latter only requires a 5'-OH group on a nucleotide and a 3' esterified phosphate group (Richardson, 1965). Thus gp pseT is capable of shuttling phosphate groups from the 3' phosphoryl to 5'-OH groups on DNA molecules (Sirotkin et al, 1978). These 2 functions are non-essential on normal laboratory strains, but, as uninfected E.coli possesses 3' phosphatase activities such as exonuclease III (Richardson and Kornberg, 1964), an in vivo role seems likely. However, there is no obvious way in which 3' phosphoryl groups could be produced in E.coli (Koerner, 1970), although the presence of such groups would certainly have drastic effects on DNA replication and recombination as they inhibit enzymes, such as DNA polymerase, involved in DNA metabolism (Goulian et al, 1968). pseT$^-$ mutants reproduce very poorly on E.coli Lit mutants whereas pseT$^+$ phage growth is normal, suggesting that E.coli has a function that can substitute for gp pseT (Cooley et al, 1979). Lit mutants seem defective in a function involved in the metabolism of nicks and single stranded gaps in DNA molecules, but retain 3' phosphatase activity. gp pseT is necessary for efficient true-late gene expression of Lit$^-$ hosts, a process which also seems to depend on the presence of nicks in template DNA (Riva et al, 1970), suggesting that gp pseT may play a role in the metabolism of such nicks. gp pseT has an apparent Mr of 33,000 (Lillehaug, 1978) and pseT is under early or quasi-late control (Wood and Revel, 1976).

(i) cd

gp cd is the phage encoded deoxycytidylate deaminase which catalyses the formation of dUMP from dCMP (Hall et al, 1967). Again this gene product is non-essential in normal laboratory strains of E.coli, but probably helps maintain good levels of dUMP after host shut off. cd is subject to early or quasi-late control (Wood and Revel, 1976). The molecular weight of gp cd is unknown.

(j) g 31

gp 31, Mr 16,000 (Castillo et al, 1977), is essential for the formation of the prehead structure in T4 head maturation (Edgar and Lielausis, 1968; Laemmli et al, 1970). It acts catalytically and does not have a structural role in the completed head. g 31 mutants accumulate 'polyheads' consisting of random aggregates of the major head subunit (gp 23), indicating that gp 31 is necessary for the ordered assembly of these subunits (Laemmli et al, 1970). gp 31 interacts with several host gene products in head assembly including the products of the groE and mop genes (Takano and Kakefuda, 1972; Georgopolous et al, 1972). groE also interacts with the λ E and B gene products (Georgopolous et al, 1972). g 31 is subject to early or quasi-late control (Wood and Revel, 1976).

(k) rIII

rIII is a member of the well known rapid lysis genes, mutants of which produce characteristic large, clear plaques (Edgar et al, 1962). The rII gene products are membrane binding proteins and rII mutations lead to the production of defective products which cause membrane abnormalities resulting in premature lysis (Ennis and Kievitt, 1973; Peterson et al, 1972; Weintraub and Frankel, 1972). rIII has not been as well studied as the related rIIA and rIIB genes,

but is thought to play a similar role. The map location of rIII has been recently revised (Revel and Leilausis, 1978). The size of gp rIII is unknown and rIII is under early or quasi-late control (Wood and Revel, 1976).

(l) g 30

gp 30, Mr 68,000 (O'Farrell et al, 1973) is the phage encoded ATP dependent DNA ligase (Fareed and Richardson, 1967) and is an essential component of the DNA replication and recombination apparatus (Broker and Doermann, 1975). It acts by catalysing the formation of phosphodiester bonds between adjacent 3'-OH and 5' phosphoryl groups on double stranded DNA molecules (Lehman, 1974). Unlike the NAD dependent DNA ligase of E.coli, gp 30 can catalyse the joining of blunt ended DNA molecules (Sgaramella et al, 1970; Sgaramella and Khorana 1972; Sugino et al, 1977). Although gp 30 is essential for T4 development in normal laboratory strains of E.coli, g 30 mutants can grow on strains of E.coli that overproduce DNA ligase.

(m) Deletions in the g 32-g 30 region

T4 mutants deleted for non-essential functions can be isolated by selecting for phage carrying a compensatory duplication of the rII genes. Such phages must undergo deletion events to comply with the headful packaging mode of T4 (Homyk and Weil, 1974). A series of deletions between g 63 and g 32 have been isolated in this way, analysed genetically and biochemically and sized by heteroduplex analysis (Homyk and Weil, 1974). For instance del (63-32), covers at least part of the td and denA genes and all of the nrdA and B genes, on the basis of tests for gene product activity.

Deletions involving g 30 have been isolated (G.G. Wilson, pers. comm.) and mutants carrying such deletions can be propagated on E.coli strains that overproduce DNA ligase. These deletions have also been analysed genetically and physically by heteroduplex analysis.

The application of restriction endonuclease technology to the analysis of the T4 genome

Wild type T4 DNA is refractory to the action of commonly used restriction endonucleases due to the present of glucosylated HMC residues (Kaplan and Nierlich, 1975). This phenomenon is overcome if either the DNA is non-glucosylated or at least some of the HMC residues are replaced by cytosine. Cytosine residues are not substrates for the T4 glucosyl transferase system.

T4 HMC DNA lacking in glucosylation can be prepared in two ways; using a phage mutant in the structural genes for the α- and β-glucosyl transferases, αgt and βgt (Georgopolous, 1967; 1968), or using an E.coli host deficient in UDP glucose pyrophosphorylase (galU) and hence the UDP glucose used in the glucosylation reaction (Hattman and Fukasawa, 1963; Shedlovsky and Brenner, 1963). Glucosylation protects T4 DNA from restriction by the rgl system of E.coli and the phage P1 system (Revel and Luria, 1970). Non-glucosylated T4 DNA is partially susceptible to digestion by R.EcoRI, but to no other restriction endonuclease tested (Kaplan and Nierlich, 1975). Presumably HMC residues interfere with recognition if they lie within, or very close to, a restriction site.

T4 DNA containing cytosine can be prepared in several ways. A temperature sensitive mutation in g 56 forms DNA completely free of cytosine at 37°C, confers lethality at 42°C, but at 39°C produces viable phage containing DNA in which 20% of the HMC residues are replaced by cytosine (Kutter and Wiberg, 1969). DNA replication and late transcription have odd kinetics in this mutant. T4 mutants of genotype g 42^-, g 56^-, g 46^-, g 47^- on non-permissive hosts produce DNA in which cytosine completely replaces HMC

(so called 100% cytosine DNA) but is fragmented by the T4 endonucleases specific for cytosine containing DNA (Kutter and Wiberg, 1968). Mutations producing deficiencies in these endonucleases coupled with mutations in g 42 and g 56, e.g. g 42^-, g 56^-, denA, denB phage, produce undegraded "100% cytosine" DNA on non-permissive hosts, but are not viable due to deficient true-late transcription (Snyder et al, 1976) although such DNA can be isolated from such infections and used for restriction analysis and molecular cloning experiments (Mattson et al, 1977). However occasional plaques are seen when such phage are plated on non-permissive hosts. These "pseudorevertants" acquire a mutation in another gene, alc, that enables such phage to express late functions from cytosine containing templates (Snyder et al, 1976). DNA replication and true-late transcription are abnormal in such phage and their burst size is reduced. Phages of genotype g 56^-, denB, alc are viable on a suppressor-free host and can contain anything up to 95% cytosine DNA (Snyder et al, 1976), whereas phages of genotype g 42^-, g 56^-, denB, alc are viable on suppressor-free hosts and have 100% cytosine DNA (Morton et al, 1978; Wilson et al, 1977). The denB mutation alone ensures that cytosine containing DNA is not degraded, indicating that although the denA product is the major endonuclease in the breakdown of host DNA, the denB product is of prime importance in the breakdown of cytosine containing T4 DNA (Kutter et al, 1975).

T4 DNA containing "20% cytosine" is susceptible to cleavage by R.EcoRI, but not R.HindIII. DNA containing greater cytosine percentages is partially digested by at least R.EcoRI and R.HindIII (Velten et al, 1976; Wilson et al, 1977; Tikhomirova et al, 1977) and 100% cytosine DNA is susceptible to all restriction endonucleases

so far tried (see, for example, Fukada et al, 1980a; Hangii and Zachau, 1980; Mileham et al, 1980). Digestion of DNA containing both cytosine and HMC is partial because the presence of HMC at restriction targets seems to protect against cleavage at that site, suggesting that if HMC is inserted into T4 DNA at random, all possible partial and single digest products should be present in digests of DNA of this sort, especially if the HMC:cytosine ratio is about 1. These modifications of T4 DNA allows its molecular cloning and the positioning of restriction targets on physical maps of the genome.

Such mapping augments genetic methods as markers that are separated by a restriction target can be unambiguously ordered in a manner analogous to deletion mapping (see Mattson et al, 1977). It is also more accurate than heteroduplex mapping, which is limited by the resolution of electron microscopy, and can ultimately be finalised by DNA sequence analysis. Cloned T4 DNA fragments can be analysed for their genetic content by marker rescue, complementation and hybridsation tests and used in functional studies of T4 genes and the controls that they are subject to, in the absence of other T4 functions that could confuse the issue. Isolated T4 DNA fragments can also be functionally analysed using transformation assays (Wain and Goldberg, 1969) and in vitro transcription/ translation systems (Hangii and Zachau, 1980).

(a) Molecular cloning of the T4 genome

Molecular cloning of the T4 genome can greatly assist the study of the control of gene expression, intrinsically interesting gene products and DNA sequences and provide improved sources of biochemically important T4 enzymes.

Successful cloning of T4 DNA was first reported by Velten et al (1976).

They were specifically trying to clone the T4 tRNA genes with a view to determining the nucleotide sequence of the region. These workers identified two R.EcoRI fragments (5.16 and 0.52 kb) that hybridised to radio-labelled T4 tRNA and were able to isolate λ recombinants containing the 0.52 kb fragment readily by a plaque hybridisation test. They established that the coding sequences for T4 RNA species I and II and $tRNA^{Arg}$ were on this fragment using radio-labelled pure T4 RNA species as probes. However they were unable to clone the 5.16 kb fragment or an R.HindIII fragment also shown to hybridise to the tRNA probe in either λ or plasmid vectors This implied that the HindIII and 5.16 kb EcoRI fragments carry a function deleterious to the host and λ.

This work has continued and recently, Fukada et al (1980b) have reported the cloning of the 5.16 kb EcoRI fragment. This fragment was isolated from agarose gels, purified and ligated into a λ vector. Prolonged incubation of transfection plates revealed the presence of tiny plaques which gave off faster growing variants and these proved to be recombinants carrying the 5.16 kb EcoRI fragment. Three classes of recombinants were found in which the fragment was inserted in either orientation; class I had undergone a deletion event in a particular region of the fragment and the fragment could subsequently be transferred to plasmid vectors; class II had an insertion or duplication in the phage right arm, which enabled the recombinant to grow faster, although no convincing explanation for this phenomenon could be found; class III contained niether insertion nor deletions, grew very slowly and generated class I deletion recombinants. The fragment in class III recombinants here could not be transferred to plasmid vectors.

These results were consistent with the notion that the 5.16 kb fragment carried a lethal function which the authors suggested from the region of the T4 genome deleted from class I recombinants could be the ipI gene. gp ipI is a small DNA binding protein (Isobe et al, 1977) and so could interfere with both host and λ DNA replication if expressed. A promotor for the tRNA genes was identified in the region deleted in class I recombinants, from gene expression and RNA polymerase binding studies.

They found that the cloned tRNA genes were initially transcribed into a polycistronic RNA precursor which is processed with $tRNA^{Glu}$, $tRNA^{Gly}$, $tRNA^{Leu}$ and $tRNA^{Thr}$ by the host, suggesting that this processing also occurs during T4 infections. However $tRNA^{Ile}$, $tRNA^{Pro}$ and $tRNA^{Ser}$, which require the T4 mb function for their stability or synthesis (Wilson and Abelson, 1972), were not expressed in the recombinants. The cloning of the mb gene could now elucidate this relationship. The authors also presented evidence for the presence of the g 57/g 1 promotor on the 5.16 kb fragment.

Fukada et al (1980a) have produced a restriction map of the T4 tRNA region and positioned the individual tRNA genes within it by hybridising appropriately digested DNA from the region, with radio-labelled pure T4 tRNA species. This has facilitated the DNA sequencing of 5.16 kb fragment (Fukada and Abelson, 1980) and the proposal that a host endonuclease is involved in T4 tRNA processing, by cleaving at unique sites as the primary transcript, to generate monomeric and dimeric tRNA precursors.

This work constitutes the most detailed analysis of a region of the T4 genome so far reported.

Abelson's group has also reported the cloning of other T4 DNA fragments generated by R.EcoRI, R.HindIII and R.PstI in λ and plasmid vectors (Velten and Abelson, 1980). Marker rescue analyses have demonstrated the presence of many T4 genes amongst these recombinants and gene expression studies have shown that g 30, g 39, g 44 and g 46 are expressed in the recombinants. The authors noted a bias against the cloning of early genes, previously noted by Wilson et al (1977). Such genes are expressed using the normal E.coli machinery and encode functions involved in DNA metabolism and host shut off, which would certainly be detrimental to the host. Thus recovery of many early T4 genes, on λ or plasmid vectors, would not be expected. On the other hand, late genes require RNA polymerase modifications for their expression, and as long as they are not expressed, such genes can be cloned even if they code for functions detrimental to the host.

Tikhomirova et al (1977) reported the isolation of λ/T4 recombinants using R.EcoRI generated fragments of non-glucosylated T4 DNA. They recovered at least seven different EcoRI fragments and demonstrated that they carried genes 26, 27, 51, 4, 5 and 50 by marker rescue analysis. Recently this group has reported the presence of at least 38 T4 genes on λ recombinants, generated by R.EcoRI digestion of 100% cytosine DNA, by marker rescue analysis (Vorozheikina et al, 1980). The authors also report that certain early, quasi-late and true-late genes can be expressed in λ recombinants, from in vitro and in vivo complementation assays, but have not unambiguously demonstrated such expression using polypeptide labelling experiments in λT4 infected cells.

Mattson et al (1977) isolated recombinant plasmids containing fragments of T4 100% cytosine DNA generated by R.EcoRI, using a colony hybridisation test against radiolabelled early or late T4 mRNA. The

presence of several T4 genes within such recombinants was confirmed by marker rescue tests. The authors suggested that a high efficiency of plating (about 1) of a T4 amber mutant on a non-permissive host carrying a recombinant plasmid, was indicative of complementation; that is not only is an intact copy of the gene in question present on the recombinant plasmid, but it is also expressed in the absence of other T4 functions. Indeed this behaviour was only seen if the recombinant plasmid carried an intact T4 gene. The only intact gene cloned that did not show complementation ability was g 22 which is a late gene and so normally requires modifications to the host transcription system for expression.

Selzer et al (1978) identified a 4.5 kb EcoRI T4 DNA fragment carrying the rIIA and rIIB genes using a transformation assay of isolated EcoRI fragments. They were unable to recover viable plasmid recombinants carrying this fragment, although they could demonstrate appropriately sized recombinant plasmids in the ligation reaction. This indicated that this fragment encodes a function deleterious to the host. To circumvent this problem, the authors cut the 4.5 kb fragment, with R.HindIII and inserted both HindIII and HindIII, EcoRI fragments into plasmid vectors. 4 groups of recombinant plasmids were recovered, on the basis of marker resuce tests: those that carried some rIIA and some g 60 markers; those that carry some rIIA markers; those that carried some rIIA and some rIIB markers; and those that carried some rIIB and some g 52 markers. Recombinants in the latter category carry the saΔ9 deletion used to remove the denB function of the parental T4 strain (Depew et al, 1975). Restriction analysis showed that these 4 groups tallied with the presence of 4 different, but contiguous T4 DNA fragments, collectively forming the 4.5 kb EcoRI fragment.

Plasmidsbelonging to the group carrying some rIIA and g 60 markers seem to express an encoded T4 function since labelled RNA isolated from E.coli cells hosting such plasmids, hybridises to T4 DNA. Such cells grow 40% more slowly than identical cells hosting plasmids of one of the other 3 groups, indicating the presence of a deleterious function in such cells. The authors suggest that such plasmids express part of the rIIA gene from an included promotor and that this truncated product is detrimental to the host. They argue that the expression of intact rIIA or rIIB genes is lethal to the host, hence their inability to clone the 4.5 kb EcoRI fragment intact. The deleterious effect of the proposed truncated rIIA product could be due to the retention of a membrane binding activity (Ennis and Kievitt, 1973) which causes a detrimental membrane association.

Wilson et al (1977) inserted several EcoRI fragments of non-glucosylated T4 DNA into λ vectors. These recombinants were analysed genetically by marker rescue analysis, which revealed the presence of markers from 31 T4 genes. These authors demonstrated that intact copies of genes containing targets for the restriction endonuclease used to generate the recombinants, can be recovered if restriction of the donor DNA is partial (non-glucosylated T4 DNA can only be digested partially). This work also produced the first restriction map of an extensive region of the T4 genome. The analysis of partially digested recombinants carrying overlapping sections of the g 50-g 20 region of the genome (about 25 kb; see Figure 1.2), allowed the ordering of EcoRI fragments within the region and marker rescue analysis of the recombinants allowed alignment of the genetic and physical maps of the region.

Wilson and Murray (1979) reported the cloning of the intact and functional T4 DNA ligase gene (g 30), using 50% cytosine DNA restricted with either R.EcoRI or R.HindIII and λ vectors. λT4 lig phages were isolated either by the ability of Red$^-$ λ recombinants to form plaques on a lig ts host at 37^{o}C (Konrad et al, 1973; Gottesman et al, 1973), the ability of integration proficient λ recombinants to form lysogens on a lig ts host at 42^{o}C, or the ability of integration proficient λ recombinants to form large turbid plaques on a lig ts host at 37^{o}C against a background of small clear plaques. All recombinants, when plated onto a suppressor free host, non-permissive for the λ recombinants, along with g 30 amber mutants, allowed the g 30 ambers to plate at an efficiency of 1-10^{-1}, which, the authors argued, was indicative of functional g 30 expression from the λ recombinant. Lysogens of such recombinants also supported the growth of a T4 g 30 deletion mutant, but did not yield wild type T4 progeny indicating that at least one end of the deletion lies outside the DNA content of the recombinants. Restriction analysis showed that g 30 lies within a 1.9 kb HindIII fragment and 3 EcoRI fragments (0.4, 0.5 and 2.2 kb). While g 30 can only be expressed from λ promotors in recombinants carrying the 1.9 kb HindIII fragment, recombinants carrying all 3 EcoRI fragments also carry a T4 promotor able to initiate g 30 expression. This promotor is also present on the 1.9 kb HindIII fragment, but for unknown reasons, is non-functional.

Recombinants carrying the 3 EcoRI fragments in both possible orientations coded for a polypeptide of 59,000 d and which is missing in g 30 amber infections. Such experiments also confirmed that the direction of transcription of g 30 is anticlockwise with respect to

the T4 map (Wood and Revel, 1976), as expected for an early gene. It seems that transcription starts within the 0.4 kb EcoRI fragment and proceeds into the 2.2 kb fragment.

Murray et al (1979) demonstrated that active ATP dependant DNA ligase can be isolated from E.coli cells lysogenic for λT4 lig phage. Such lysogens are valuable sources of T4 DNA ligase since they provide a genetic means of purification from other T4 proteins such as nucleases and facilitate the amplification of DNA ligase expression. T4 DNA ligase prepared in this way is capable of catalysing blunt end ligation with good efficiency.

Revel (manuscript in preparation) has cloned the T4 DNA carrying the g 34-g 38 region in λ vectors. The presence of these genes, whose products are involved in the production of tail fibres, was shown by marker rescue, and restriction analysis of the recombinant DNAs showed that the region is contained within 6 HindIII and 6 EcoRI fragments. A restriction map of the region was deduced from marker rescue analyses of cloned fragments using genetic markers within the region. gps 34, 35, 37 and 38 are synthesised by these recombinants as are two other polypeptides whose nature is unknown. gp 36 was not detected in such experiments, for reasons that were not apparent. An in vivo complementation test suggested that gp 34 is functionally active in λ recombinants.

Mileham et al (1980) reported the cloning and organisation of the frd-DNA ligase region of the T4 genome, which is presented in detail in this thesis.

(b) Restriction mapping of the T4 genome

Early restriction mapping of T4 was limited to placing restriction sites contained within cloned T4 fragments whose genetic content was known (Mattson et al, 1977; Velten et al, 1976; Wilson et al, 1977). This type of data has accumulated to the extent that most of the genome is covered by such maps (see for example, Velten and Abelson, 1980; Mileham et al, 1980).

Several groups have constructed T4 restriction maps on a purely physical basis. Takahashi et al (1979) reported that while T4 "100% cytosine" DNA contained 8 R.SalI sites it only contained a single R.BamH1 site, which, they pointed out, would be an ideal reference point for restriction maps. R.BamH1 sites had been looked for previously, but not found (Wilson et al, 1977), as the presence of the unique R.BamH1 site is only revealed in double digest reactions of the sort used by Takahashi et al (1979). The position of this R.BamH1 site was found to be within an 11.5 kb HindIII that spans genes 6 to 12 and a 3.7 kb EcoRI fragment known to contain g 8 and parts of genes 7 and 9 (this Thesis; V. Tanyashin, pers. comm.) and has now been placed within, or very near to, g 8 (Wilson et al, 1980).

The first complete restriction maps were deduced by Kiko et al (1979), who used partial and double digest data to construct complete maps for R.SmaI and R.KpnI and an almost complete R.BglII map. Ruger et al (1979), later completed this R.BglII map and constructed complete R.SalI and R.XhoI maps using partial, and multiple digest data. Carlson and Nicolaisen (1979) later published complete R.SalI and R.KpnI maps which were essentially in agreement with those published previously. The only real discrepancy is the absence of a 3.3 kb KpnI fragment from the Carlson and Nicolaisen map.

Kutter and O'Farrell (pers. comm.) are constructing restriction maps for several enzymes such as R.EcoRI, R.HindIII and R.PstI using a 2 dimensional gel technique and these are nearing completion.

T4 DNA fragments can also be analysed by in vitro transcription-translation systems, provided that they code for an identifiable function. Hangii and Zachau (1980) have used such a system to analyse the structure of the T4 genome around the frd gene and have constructed a restriction map of the region using many enzymes. The authors positioned the genes 32, td and frd within their map by assaying the ability of T4 DNA cut with particular restriction endonucleases to produce either functional gp frd or radio-labelled gp 32 in the in vitro transcription-translation system, and by hybridisation of appropriately restricted 100% cytosine against a T4 DNA radio-labelled probe to a λT4 recombinant carrying the T4 td gene described by Mileham et al (1980). This method has shortcomings as the gene assayed must be linked to a promotor that can function in the in vitro system in order to be expressed.

(c) Some uses of cloned T4 fragments

Cloned T4 fragments should provide useful material for a variety of molecular studies of T4 functions including the control of initiation and termination of transcription of the different classes of T4 genes, for genetic mapping and for the production of T4 encoded enzymes as shown by Murray et al (1980).

Halpern et al (1979) used various T4 plasmid clones to screen for the T4 origins of replication and discovered that replication proceeds bidirectionally from at least one major origin in the g 50-g 5 region and one minor origin in the gw-g 29 region. The search for

T4 origins of replication had previously given amibugous results due to the asynchrony of T4 replication (Kozinski and Doerman, 1975).

Volker and Showe (1980) used T4 plasmid clones to specifically mutagenise a region of the T4 genome (g 20-g 21) *in vitro*. They recovered T4 phage carrying conditionally lethal mutations in g 20 and g 21 by recombination between wild type T4 and mutagenised recombinant plasmids in *E.coli* cells. The authors suggest that this approach provides a means of saturating a particular gene with mutations, so rendering essential T4 genes available to the types of fine structure mapping previously possible for non-essential genes such as the *rII* genes (Benzer, 1961). Gene products whose structural gene has many genetic markers are excellent subjects for detailed functional studies.

Cloned T4 fragments are useful in genetic mapping as they carry a defined region of the T4 genome. Mattson *et al* (1977) were able to position a g 41 mutation relative to others and correct the order of two g 43 mutations. The use of cloned fragments overcomes the ambiguities of conventional two and three factor crosses and should be especially useful for essential genes where deletion mutations are not available.

(d) Vectors

3 basic types of vectors are available for the molecular cloning of prokaryotic DNA; bacteriophages, commonly λ (see Murray, 1978), plasmids (see Sutcliffe and Ausubel, 1978) and 'cosmids' which combine desirable features of λ and plasmid vectors (Collins and Hohn, 1978). Only the first two types will be considered here, both of which have advantages and disadvantages when applied to T4 cloning.

Plasmid vectors have several useful characteristics (Helinski et al, 1977; Sutcliffe and Ausubel, 1978): at least one positive selectable marker, usually an antibiotic resistance; have a relaxed mode of replication and so are present in several copies per cell; have unique sites for several restriction endonucleases, most of which are within the structural gene for a function that can be readily screened, usually an antibiotic resistance gene, thus making rapid screening of putative recombinants possible by insertional inactivation of the gene in question. Their DNA can be amplified in the presence of chloramphenicol to allow the plasmid copy number per cell to exceed 1000 and enabling efficient recovery of plasmid DNA; and can incorporate large fragments of donor DNA, for instance an F factor fragment carrying the entire tra operon of 40 kb has been cloned in plasmid vectors (Johnson and Willetts, 1980). However, the efficiency of recovery of such large plasmids by transformation is very low, indeed the recovery of the recombinant plasmid carrying the F factor tra operon was only possible due to powerful selection (Johnson and Willetts, 1980). If a cloned gene on a recombinant plasmid is expressed in the host, the gene product must not be detrimental to the host if it is to remain viable. Thus genes whose functions interfere with the host cannot be cloned on plasmid vectors, and these include many phage genes, such as membrane binding proteins like the rII gene products of T4 demonstrated by Selzer et al (1978). Such deleterious host effects may be due to the increased gene dosage supplied by relaxed plasmids, for example the wild type polA gene of E.coli cannot be cloned on plasmid vectors along with its own promotor (Kelley et al, 1977).

Phage λ can act as an effective cloning vector as its mode of

packaging allows viable λ chromosomes to range from 75 to 109% of the wild type length (see Thomas et al, 1974). The deletion of non-essential regions of the λ chromosome reduces its size and allows the incorporation of donor DNA fragments of an increased size. The present λ vectors can accommodate donor DNA fragments of up to 20 kb.

λ vectors are of two basic types; insertion and replacement vectors (see Murray, 1978). Insertion vectors have a unique site for a particular restriction endonuclease, quite often located within the cI gene, so that when donor DNA, cut with the same enzyme, is inserted into the λ chromosome, the resulting recombinant phage have a clear plaque phenotype which enables rapid screening. Replacement vectors typically carry a fragment of foreign DNA coding for a readily identifiable function, e.g. the lacZ gene, bounded at each end by restriction sites for a particular restriction endonuclease. When such a phage is digested with the appropriate restriction endonuclease, this fragment is cut out and can be replaced by a fragment of donor DNA generated with the same enzyme. Putative recombinants here can be rapidly screened by the loss of function encoded by the fragment replaced. Cloned DNA can be readily amplified by the phage infectious process, which results in high titres of phage and large amounts of DNA. λ vectors are only commonly available for use with R.EcoRI, R.HindIII and R.BamH1, although vectors for use with R.SalI, R.SstI, R.XhoI, R.XmaI and R.SmaI exist. As the λ lytic cycle is a terminal event resulting in host death, many genes whose products cannot be tolerated in living host cells, can be cloned in a functional form in λ vectors e.g. the E.coli polA gene (Kelley et al, 1977). However genes whose products interfere with λ development cannot be cloned in λ vectors.

(e) Amplification of gene expression

Both plasmid and phage λ cloning systems have the potential to amplify the expression of cloned genes. Many plasmid vectors replicate in a relaxed mode and so are present at a copy number of about 20 per chromosome. This increase in copy number of cloned genes is not necessarily sufficient to alleviate the action of repressor molecules by titration and so host cells must either have defective repressors, or be induced to allow the maximal expression of cloned repressible genes. For example the enzymes involved in tryptophan synthesis comprise 20-40% of the total soluble protein after derepression of cells hosting recombinant plasmids carrying the trp operon (Helinski et al, 1977). An attempt to achieve amplification of the trp gene products in a trpR$^-$ host was less successful as the overproduction of these enzymes proved lethal to the host cells.

Amplification of gene expression using λ vectors can be achieved in several ways but must at least involve preventing lysis of the infected cell while allowing continued DNA replication (see Murray, 1978). Mutations in the S gene (lysis function) were first used to satisfy these requirements as they allow DNA replication and protein synthesis to continue for several hours after infection, in the absence of lysis. Mutations in the Q gene, which controls rightward transcription from P'_R, block late λ gene expression. Thus transcripts initiated from other promotors are translated more efficiently at late times in λ infection, due to the lack of competition by P'_R initiated transcripts. λtrp, Q$^-$, S$^-$ phage carrying the structural genes for anthranilate synthetase, trpE and D, produce this protein to such an extent that it comprises more than

25% of the soluble protein of infected cells. Amplification of such recombinant phage DNA titrates away the *trp* repressor, thus allowing full expression of the *trp* operon from its own promotor. Any lethal effects of high concentrations of particular gene products, such as the enzymes of tryptophan synthesis, is less important here as phage infection itself results in cell death.

The linking of transcription of a cloned gene to a major λ promotor offers several advantages in expression amplification: expression of cloned genes here is independent of the normal control systems of the genes such as repression, attenuation , induction or autogenous control; and expression of cloned genes is subject to normal λ control, thus expression is repressed in the lysogenic state, but allowed in the lytic cycle and increased with specific modifications. An example of the later is the removal of the *cro* gene product which negatively controls the λ lytic cycle by blocking transcription from P_L. Phage of type *cro*$^-$, *Q*$^-$, *S*$^-$ are transcribed exclusively from P_L. However, *cro*$^-$ phage are difficult to construct and propagate, a difficulty that is overcome by the use of *cro*^{ts} mutants which propagate normally at permissive temperatures or hybrid immunity phage (see Murray, 1977).

Plasmids that carry either the control region or P_L promotor of phage λ, offer an attractive combination of desirable features of both plasmid and λ cloning vehicles with respect to the amplification of gene products (Bernard *et al*, 1979; Helinski *et al*, 1977). This system is mainly applicable to cloned DNA fragments carrying a desirable gene, but lacking a promotor. λ promotors carried by such plasmids are normally repressed by the *cI* gene product which is either supplied by a linked *cI* gene or an unlinked *cI* gene carried

on a prophage within the cell, and so cloned genes served by the λ promotor are not expressed. However the cI allele involved codes for a temperature sensitive repressor, thus repression is alleviated at the non-permissive temperature allowing maximal expression of cloned genes linked to the λ promotors. Prophages that supply the cI function here also carry an N^- mutation to prevent λ development.

Aims and Strategies

The aim of the work presented in this thesis was the molecular cloning of the region of T4 genome containing the genes coding for polynucleotide kinase (pseT) and RNA ligase (g 63), with a view to achieving amplification of their products, which are useful enzymes in biochemical research. Marker rescue tests had failed to detect the presence of g 63 in either plasmid or λ recombinants and are not applicable to the pseT gene whose product is non-essential for T4 development. Thus an indirect method of cloning these genes was sought. Recombinants carrying genes on either side of the region of interest and whose presence could be directly selected, would facilitate the isolation of recombinants carrying DNA fragments from this region.

λT4 recombinants carrying g 30, which lies about 10 kb anticlockwise of genes 63 and pseT on the T4 map (Wood and Revel, 1976; see Figure 1.2), were isolated just before the start of this project. Also λ recombinants carrying the thymidylate synthetase gene (thyA) from E.coli (Borck et al, 1976) and plasmid derivatives carrying the B.subtilis phage Phi-3-T thymidylate synthetase gene (Ehrlich et al, 1977) had been isolated by their ability to complement E.coli thyA hosts, and so it seemed probable that recombinants carrying the T4

thymidylate synthetase gene (td), which maps about 10 kb clockwise of genes 63 and pseT on the T4 map (Wood and Revel, 1976; see Figure 1.2), could be detected similarly. The cloning of DNA on either side of genes 63 and pseT could fortuitously lead to the isolation of recombinants carrying either gene and would certainly provide probes for the identification of recombinants containing overlapping fragments in the direction of these genes.

The use of partially digested donor DNA in the generation of recombinants, enables contiguous fragments of donor DNA to be incorporated into a single vector, thus increasing the capacity of single recombinants to carry unselected markers, and allowing the recovery of genes containing sites for the restriction endonuclease used in generating the recombinants (Wilson et al, 1977). Thus the T4 DNA used in cloning experiments here was either non-glucosylated or 50% cytosine DNA so that when the former was digested with R.EcoRI or the latter with R.EcoRI or R.HindIII, the resultant DNA fragments would necessarily be partial products.

λ vectors were chosen for the basic cloning experiments as it seemed logical that certain phage functions would not be tolerated in plasmid vectors, but could be tolerated in phage vectors. This is especially the case for many early T4 genes as they are involved in processes such as DNA metabolism and are readily expressed. Indeed the region of the T4 genome under consideration here, contains many early genes. Plasmid vectors were used to propagate particular T4 DNA fragments previously cloned in λ vectors, to provide probes to screen for λT4 recombinants carrying overlapping T4 DNA fragments.

The td-g 30 region of the T4 genome contains several known genes

whose products could conceivably interfere with the successful cloning of the entire region. gp denA is an endonuclease that specifically attacks cytosine containing DNA, which suggests that recombinants carrying an active denA gene cannot be isolated as gp denA would digest the recombinant DNA. Thus T4 DNA carrying a denA mutation was also used to generate recombinants. gp alc interacts with the host RNA polymerase so that it only transcribes DNA containing HMC and so would block the transcription of λT4 recombinant DNA carrying an active alc gene. However such problems are automatically negated by the use of cytosine containing T4 DNA in the generation of recombinants as such DNA necessarily carries an alc mutation. The rIII gene product seems to be involved in host membrane interactions and it is known that the closely related rII genes cannot be cloned intact in plasmid vectors (Selzer et al, 1978).

The only obvious remaining gene product encoded by this region of the T4 genome that could interfere with λ functions specifically, is g 31. The groE gene product of E.coli interacts with the products of the λE gene and T4 g 31 during λ and T4 head development respectively. Thus the presence of a functional copy of g 31 on a λ recombinant could lead to the impairment of λ head formation due to gp 31 competitively inhibiting the groE-λE gene interaction.

Thus while it was recognised that there may be intrinsic problems in successfully cloning the entire td-g 30 region of the T4 genome, it was hoped that considerations such as those listed above, would lead to the majority of the region being isolated on λ recombinants.

Selection of Vectors and Expression of Cloned Genes in λ Vectors

λ vectors used in the generation of the thymidylate synthetase recombinants were chosen to ensure that cloned T4 functions could be expressed from λ promoters during normal lytic growth and to be able to form lysogens, thus allowing the identification of promoters serving T4 genes present on the recombinants. λ vectors chosen here were λNM816 and 616, replacement vectors for R.EcoRI generated DNA fragments, whose replaceable DNA segment lies in the b2 region of the λ chromosome and λNM540, an insertion vector for R.HindIII generated DNA fragments, whose single R.HindIII site is shnλ3, just to the left of the attachment site on the λ chromosome (see Figure 2.1). Donor DNA inserted into these vectors can be transcribed leftwards from the P_L promoter and rightwards from the P'_R promoter during the lytic cycle (see Murray et al, 1979). All three vectors have intact attachment sites and cI genes and so form stable lysogens, at least at 32°C (λNM816 has a temperature sensitive repressor).

Vectors used in the molecular cloning of DNA fragments thought to carry deleterious functions, were chosen on the basis that they, or their derivatives, were unable to form lysogens, or only permitted low levels of expression of cloned genes from λ promoters during the lytic cycle. The expression of deleterious functions in lysogens, is likely to result in cell death, as the cell is continually affected by such functions, whereas normal, or especially low level, expression of the same functions during the lytic cycle may be tolerated. This is because the lytic cycle is a short-lived, terminal event. Recombinant phage carrying a deleterious function of this nature may be easier to isolate if they have a clear plaque

phenotype since phage with turbid phenotypes are able to form lysogens and so could produce small, irregular plaques, due to lysogen death. Thus the vectors used for this type of work were λNM762, a replacement vector for R.HindIII generated DNA fragments, whose replaceable DNA segment is in the b2 region of the λ genome, and λNM607, an insertion vector for R.EcoRI generated DNA fragments, whose single R.EcoRI site is within the cI gene (see Figure 2.1). Neither vector can form lysogens as they are deleted for their attachment sites, and their derivatives lack a functional cI gene, either by a deletion, in the case of λNM762 or by insertional inactivation in λNM607 recombinants. λ promoted expression of cloned functions in λNM762, is similar to that in λNM540, 616 and 816, whereas such expression of cloned functions in λNM607, can only be initiated from the λ pre and prm promoters. pre and prm normally serve the cI gene immediately after infection and throughout lysogeny respectively (see Reichardt, 1975). Expression, from both promoters, is blocked by the action of the cro gene product during lytic growth. Expression of cloned genes from pre and prm can only occur if the normal direction of transcription of a cloned gene is the same as that of the cI gene, that is only in one of the two possible orientations of the donor DNA insert. Thus genes cloned into λNM607 are only expressed for a short time immediately after infection, unless the recombinant phage also carries a functional promoter for the gene in question.

Foreign genes inserted into the b2 region of λ vectors are either transcribed as 'delayed-early' genes from P_L or as 'late' genes from P'_R, depending on their orientation. In λ infection, early gene products appear soon after infection and include the N

gene product which is necessary for the appearance of delayed-early gene products (see Echols, 1972). The product of the delayed-early λ gene Q, acts as a positive regulator of late gene expression by allowing the initiation of transcription from P'_R. Thus cloned gene products appear at early times after infection, if their structural gene is transcribed from P_L, but only at late times, if their structural gene is transcribed from P'_R.

2. MATERIALS AND METHODS

1. Materials

(a) Media

Phage Buffer

Potassium dihydrogen phosphate	3 g
di-Sodium hydrogen phosphate (anhydrous)	7 g
Sodium chloride	5 g
Magnesium sulphate	10 ml of 0.1 M
Calcium chloride	10 ml of 0.01 M
Gelatin	1 ml of 1% w/v
Distilled water	to 1 litre

L-broth

Difco Bacto Tryptone	10 g
Difco Bacto Yeast Extract	5 g
Sodium chloride	10 g
Distilled water	to 1 litre

This was adjusted to pH 7.2 with sodium hydroxide before autoclaving. L-broth was supplemented with thymine, at 40 µg/ml for the growth of strains carrying the thyA mutation, ampicillin at 40 µg/ml for the growth of strains carrying plasmids which code for ampicillin resistance and chloramphenicol at 100 µg/ml for the amplification of plasmid DNA in chloramphenicol-sensitive hosts.

5 x Spizizen Salts

Ammonium sulphate	10 g
di-Potassium hydrogen phosphate	70 g
Potassium dihydrogen phosphate	30 g
tri-Sodium citrate. 2 H_2O	5 g
Magnesium sulphate. 7 H_2O	1 g
Distilled water	to 1 litre

BBL Agar

Tripticase (Baltimore Biological Labs)	10 g
Sodium chloride	5 g
Distilled water	to 1 litre

Bottom layer agar included 10 g/l Difco Bacto Agar, whereas top layer agar included 6.5 g/l of the agar.

L-Agar

Difco Bacto Tryptone	10 g
Difco Bacto Yeast Extract	5 g
Sodium chloride	10 g
Difco Bacto Agar	15 g
Distilled water	to 1 litre

The pH was adjusted to pH 7.2 with sodium hydroxide prior to autoclaving. This medium was supplemented with thymine at 40 µg/ml, for the growth of strains carrying the thyA mutation, chloramphenicol and ampicillin at 100 µg/ml and tetracycline at 50 µg/ml, for the growth of strains carrying plasmids specifying resistance to these antibiotics.

Minimal Agar

Davis New Zealand Agar	16 g
Distilled water	to 800 ml

200 ml of sterile 5x Spizizen salts were added after autoclaving. The following supplements were added when required:

Glucose	to 0.2%
Amino acids	20 µg/ml
Difco Bacto Casamino acids	to 0.05%
Thymine	40 µg/ml
Thiamine	2 µg/ml
Trimethoprim	0.5-1.0 µg/ml

4 x M9 salts

di-Sodium hydrogen phosphate	28 g
Potassium dihydrogen phosphate	12 g
Sodium chloride	2 g
Ammonium chloride	4 g
Distilled water	to 1 litre

M9-maltose medium

4 x M9 salts	250 ml
20% maltose	15 ml
Distilled water	to 1 litre

Minimal Top Layer Agar

Davis New Zealand Agar	6.5 g
Distilled water	to 1 litre

Lactose-MacConkey Agar

Oxoid MacConkey Agar No. 3	40 g
Distilled water	to 1 litre

Xg-Indicator Agar

BBL bottom layer agar containing 40 mg/l of 5-Bromo-4 Chloro-3 Indoyl-β-D-Galactoside (Xg) purchased from Bachem Inc.

All media were sterilised by autoclaving at 15 lb/sq in for 15 minutes.

(b) Enzymes and Chemicals

R.EcoRI, R.HindIII, R.BamHI were provided by K. Mileham; R.BglIII and R.XhoI by B. Sain; R.PstI by J. de Banzie; R.XbaI, R.HaeIII and R.KpnI were purchased from New England BioLabs and R.SmaI from Boehringer. T4 DNA ligase was provided by S. Bruce and DNA polymerase I and DNAase I by D. Finnegan. Pancreatic DNAase, RNAase, lysozyme, trimethoprim and chloramphenicol were purchased from Sigma Chemical Company; ampicillin from Beecham Research Laboratories; tetracycline from Lederle Laboratories. Polyethylene glycol 6,000, acrylamide purified for gel electrophoresis, N,N'-methylene bisacrylamide, N,N,N',N'-tetramethyl-ethylene diamine, polyethyleneimine (50% solution) and sodium lauryl sulphate were purchased from BDH Ltd, and agarose from Miles Laboratories. Analar grade chemicals were used for all buffer solutions, special media and other reagents. L- ^{35}S -methionine (spec.act. 1,100 Ci/mmol; 6.85 mCi/ml), ^{32}P α-dCTP (spec.act. 400 Ci/mmol; 1 mCi/ml) and L- ^{14}C -leucine (spec.act. 0.348 Ci/mmol; 50 μCi/ml) were

purchased from the Radiochemical Centre, Amersham, UK; Nitrocellulose filters were from Schleicher and Schüll, fast tungstate intensifying screens and FP4 film from Ilford; and X-ray film (X-O mat H), from Kodak.

(c) Bacterial Strains

Strain	Genotype	Source	Reference
C600	supE tonA thr leu thi lac	NEM	Appleyard (1954)
C600(λ)	supE tonA thr leu thi lac(λ)	NEM	
C600(λimm^{21})	supE tonA thr leu thi lac (λimm^{21})	NEM	
C600(λimm^{434})	supE tonA thr leu thi lac (λimm^{434})	NEM	
594	gal str^{R} sup^{o}	NEM	Weigle (1966)
594 (λ)	gal str^{R} sup^{o} (λ)	NEM	
594 (λimm^{21})	gal str^{R} sup^{o} (λimm^{21})	NEM	
594 (λimm^{434})	gal str^{R} sup^{o} (λimm^{434})	NEM	
W3101	wild type	NEM	Campbell (1961)
W3101/λ	λ^{R} sup^{o}	AJM	
CR63	supD λ^{R}	NEM	Appleyard et al (1956)
CA7105	HfrH galU sup^{o} malA rglA	JGS	Wilson et al (1977)
ED8689	sup^{o} trpR hsdR	NEM	Wilson et al (1977)
ED8689/λ	sup^{o} trpR hsdR λ^{R}	AJM	
ED8614	thyA trpR trpBE9Δ	NEM	Borck et al (1976)
ED8654	supE supF met hsdR^{-} hsdM^{+}	NEM	Murray et al (1977)
802	hsdR metE supE rglA rglB	HRR	Wood (1966)
ED8538	tonA lacZam sup^{o} trpA str^{R}	NEM	Murray et al (1977)
NM344	hsdR^{-} hsdM^{-} lacZΔ	NEM	
HB101/λ	recA hsdS pro gal str^{R} λ^{R}	NEM	
S159	sup^{o} uvrA	NEM	Jaskunas et al (1975)
803/λ	hsdS metE supE rglA rglB λ^{R}	NEM	

NEM = N. Murray; AJM = A. Mileham; JGS = J. Scaife, HRR = H. Revel

(d) Cloning Vectors

(i) λ derivatives

Strain	Genotype	Source	Reference
NM540	srI (1,2)del i^{21} nin Q^{80}	NEM	Borck et al (1976)
NM607	b538 srI 3.0 i^{434} srI4.0 srI5.0	NEM	Murray et al (1977)
NM616	plac5 att^{+} srI3.0 i^{21} srI4.0 nin srI5.0	NEM	
NM762	srI(1,2)del supF (att-red)del cIdel KH54 nin shn6.0	NEM	Murray et al (1977)
NM816	plac5 srI3.0 i^{21ts} srI4.0 nin srI5.0	NEM	Wilson and Murray (1979)

See Figure 2.1 for diagrams of vectors.

(ii) plasmids

Plasmid	Source	Reference
pBR322	DJF	Bolivar et al (1977)
pBR325	NSW	Bolivar (1978)

A derivative of pBR313 including the DNA ligase gene of T4 (Wilson and Murray, 1979) was used as a hybridisation probe.

NEM = N. Murray; DJF = D. Finnegan; NSW = N. Willetts

(e) Phage strains

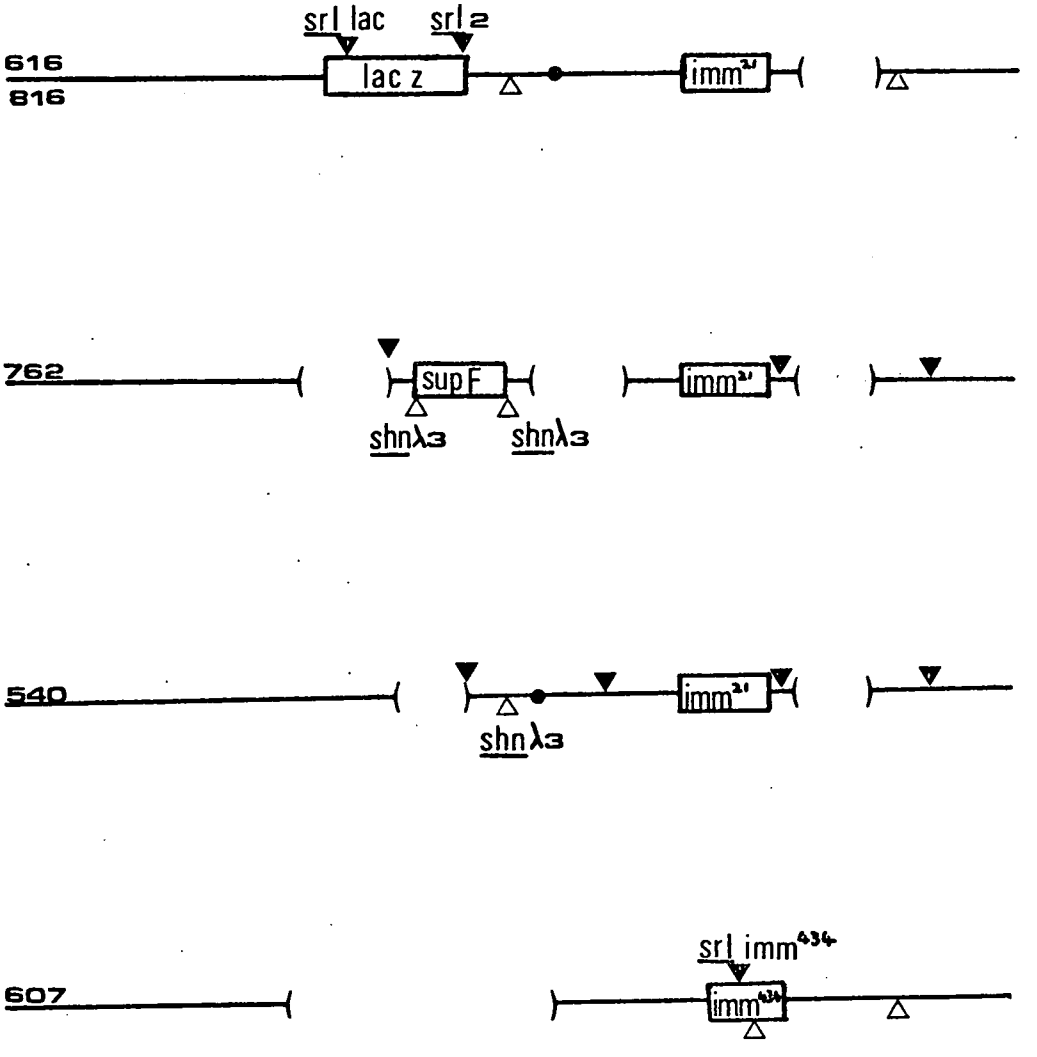
(i) λ strains

Strain	Genotype	Reference
λ^{+}	wild type	
λclear	cI26	
λvir	virulent mutation	
λimm^{434}	imm^{434}	
λimm^{434} clear	imm^{434} clear	
λimm^{21}	imm^{21}	

Figure 2.1

λ vectors. Boxes represent substitutions and brackets, the extent of deletions; ▼ represent R.EcoRI targets, Δ, R.HindIII targets and the solid circle, the λ attachment site. Dashed lines show vector DNA that is replaced in recombinant derivatives and the labelled restriction targets identify the positions at which donor DNA is inserted.

Figure 2.1


srl lac
srl 2
616
816
lac z
imm21
762
supF
shnλ3
shnλ3
imm21
540
shnλ3
imm21
srl imm434
607
imm434

Strain	Genotype	Reference
λimm^{21} clear	imm^{21} clear	
λE^{am}	E am	
λN^{am}	Nam	
NM75	b2 red3	
816-1	NM816: carrying all T4 g30	Wilson and Murray (1979)
λlig6-2	NM816: carrying part of g 30	Wilson and Murray (1979)

All λ strains were provided by N.E. Murray.

(ii) T4 strains

Strain	Genotype	Reference/Source
alc1	alc1, amE51(56), H23(denB-rII), nd28(denA); 50% cytosine DNA	E. Kutter
alc4	alc4, amE51(56), NB5060(denB-rII); 50% cytosine	Wilson et al (1977)
alc7	alc7, amE51(56), NM5060(denB-rII), amC87(42); 100% cytosine	Wilson et al (1977)
JW819	alc10, amE51(56), D292(denB), nd28(denA), amN55x5(42); 100% cytosine	J.S. Wiberg
nd28	denA	E. Kutter
T4D	wild type	N.E. Murray
pseT-1	point mutant in pseT gene	L. Snyder: Sirotkin el al (1978)
pseTΔ1	deletion mutant removing all known pseT markers	L. Snyder: Sirotkin et al (1978)
del(63-32)1	deletion between genes 63 and 32	H. Revel: Homyk and Weil (1974)
del(63-32)9	deletion between genes 63 and 32	H. Revel: Homyk and Weil (1974)

Strain	Genotype	Reference/Source
rEDdf41	rII deletion	H. Revel: Edgar et al (1962)
amB55	amber mutations in nrdB, frd	H. Revel: Yeh and Tessman (1972)

The following T4 amber mutants were used in marker rescue assays and were provided by H. Revel:

B250(g 6); B16(g 7); N132 (g 8); E17(g 9); B255(g 10); N93(g 11); N104(g 12); N54(g 31); S54(g 31); A453(g 32); M69(g 63); E1072(g 63).

(e) solutions for SDS polyacrylamide gel electrophoresis

Stock acrylamide

Acrylamide	30 g
N,N'-methylene bisacrylamide	0.8 g
Distiled water	to 100 ml

4x Upper Tris

Tris	6.06 g
10% SDS	4.0 ml

Adjust to pH 6.8 HCl and make up to 100 ml with distilled water.

4x Lower Tris

Tris	18.17 g
10%SDS	4.0 ml

Adjust to pH 6.8 with HCl and make up to 100 ml with distilled water.

Electrophoresis buffer

Tris	3 g
Glycine	14.4 g
20% SDS	10 ml
Distilled water	to 1 litre

Sample buffer

Glycerol	0.85 ml
β-mercaptoethanol (100%)	0.5 ml
10% SDS	3.0 ml
4 x Upper Tris	1.24 ml
Bromophenol blue	1 mg
Distilled water	to 10 ml

Gradient gel solutions

	10%	20%
Distilled water	9.1 ml	1.98 ml
4x Lower Tris	5.5 ml	5.5 ml
Acrylamide solution	7.33 ml	14.4 ml
10% Ammonium persulphate	20.5 µl	16 µl
N,N,N',N'-tetramethylethylenediamine (TEMED)	10.25 µl	17.6 µl

10% linear gel solutions

Distilled water	18.2 ml
4x Lower Tris	11.0 ml
Acrylamide solution	14.66 ml
10% Ammonium persulphate	41 µl
TEMED	20.5 µl

Stacking gel solution

Distilled water	6.5 ml
4x Upper Tris	2.5 ml
Acrylamide solution	1.0 ml
10% Ammonium persulphate	40 µl
TEMED	15 µl

2. Methods

(a) Plating cells

A fresh overnight bacterial culture was diluted 20-fold into L-broth and grown with shaking at the required temperature (usually 37°C), until a concentration of about 2-5 x 10^8 cells/ml was reached. The cells were then pelleted, resuspended in an equal volume of 10 mM $MgSO_4$ and stored at 4°C.

(b) Plate Lysates

A well isolated single plaque was picked into 1 ml of phage buffer containing a few drops of chloroform and shaken briefly. Between 0.1 and 0.2 ml of this phage suspension was adsorbed for a few minutes to either 0.1 ml of a fresh overnight culture for T4 strains or to 0.2 ml of plating cells for λ strains. 3 ml of BBL top layer agar was added, the mix poured onto a fresh L-agar plate and the plate incubated at the required temperature, usually 37°C. When confluent lysis was observed, usually after 4-8 hours, the top layer was harvested in 3 ml of L-broth. 0.2 ml of chloroform was then added and the suspension whirlimixed. The agar, cell debris and chloroform were pelleted and the supernatant decanted, titred and stored at 4°C. Titres were typically about 2-5 x 10^{10} pfu/ml.

(c) Phage Titrations

Phage stocks were titrated after serial dilution in phage buffer. 0.1 ml of a suitable dilution was mixed with either 0.1 ml of a fresh overnight culture, for T4 strains or 0.2 ml of plating cells for λ strains and left to adsorb for a few minutes at room temperature. 3 ml of BBL top layer agar was then added, the mix poured onto a BBL agar plate and the plate incubated overnight at the required

temperature, usually 37°C. The number of plaques on the plate next day was counted and the titre calculated.

(d) Construction of lysogenic and λ resistant derivatives

0.2 ml of plating cells of the appropriate strain was mixed with 3 ml of BBL top layer agar and poured onto BBL agar plates. A drop of the phage to be used in lysogen construction or λvir in the construction of λ resistant derivatives, at a concentration of about 10^7 pfu/ml, was applied to the centre of the plate. The plate was incubated overnight at the required temperature and a loopful of top layer from the area of lysis, streaked out on an L-agar plate next day. After incubating overnight at the desired temperature several isolated single colonies were picked into 1 ml of L-broth and grown for several hours at the same temperature. 0.2 ml of each culture was mixed with 0.3 ml 0.1 M $MgSO_4$ and 3 ml BBL top layer agar and poured onto a BBL agar plate. Spots of λvir and a homoimmune phage carrying a mutation in the cI gene at a concentration of 10^7 pfu/ml, were applied to the surface of the plate and the plate incubated at the required temperature overnight. Lysogenic derivatives were resistant to the cI^- homoimmune phage but sensitive to λvir, whereas resistant derivatives were resistant to both phage types.

(e) Tests for putative recombinants

(i) Lac phenotype

λ vectors NM616 and 816 carry the lacZ gene and so derivatives of these vectors were tested for their Lac phenotype by spotting a suitable dilution onto a lawn of a lacZΔ strain (NM344) on a Lac- indicator plate. Similarly λ vector 762 carries the supF gene and

so derivatives of this vector were tested by spotting a suitable dilution onto a lawn of a lacZ amber strain (ED8538) on a Lac- indicator plate. Lac-indicator plates were either Lac-McConkey agar or BBL agar supplemented with XG. Derivatives that were colourless on either indicator plate had lost the DNA fragment carrying either the lacZ or supF genes and were putative recombinants.

(ii) Insertional inactivation of the λ repressor gene

Derivatives of λ vector NM607 carrying a fragment of donor DNA have a clear plaque morphology due to the insertional inactivation of the λ repressor gene. Thus putative recombinants here were identified by a clear plaque morphology.

(iii) Plasmid recombinants

Putative recombinants here were screened by the insertional inactivation of the tetracycline resistance gene, in the case of pBR322 or the chloramphenicol resistance gene, in the case of pBR325.

(f) Selection of λtd$^+$ recombinants

About 1000 pfu from a pooled lysate of λT4 recombinants were mixed with 0.2 ml of ED8614 plating cells and 3 ml of BBL top layer agar and poured onto a BBL plate. The plate was incubated overnight at either 32°C or 37°C. λtd$^+$ recombinants were identified as normal sized turbid or clear plaques against a background of tiny plaques formed by other λT4 recombinants and vectors. Single plaques derived from each recombinant were plated out and single plaques from this plate retested for the ability to form plaques on ED8614 on BBL agar plates.

Derivatives of ED8614 lysogenic for λtd$^+$ phages were constructed non-selectively in the presence of 40 μg/ml of thymine. These

lysogens were then tested for thymine independence by streaking onto L-agar plates.

(g) Selection of λ*frd*$^+$ recombiants

Either about 1000 pfu from a pooled lysate of λT4 recombinants, or about 100 pfu of a purified λT4 derivative, were mixed with 0.2 ml of ED8689 cells and 3 ml of minimal top layer agar and poured onto a minimal-glucose agar plate supplemented with 0.5-1.0 µg/ml of trimethoprim. The plate was incubated overnight at 37^{o}C and any resultant plaques, either turbid or clear, were identified as λ*frd*$^+$ recombinants. Each derivative was subject to two rounds of plaque purification and retested for the ability to form plaques on ED8689 in the presence of trimethoprim.

Derivatives of ED8689 lysogenic for λ*frd*$^+$ phages were constructed in the absence of trimethoprim and then tested for trimethoprim resistance by streaking onto minimal-glucose agar plates containing 0.5-1.0 µg/ml of trimethoprim.

(h) Marker rescue tests

Marker rescue tests were performed in three basic ways:

(i) About 10^8 λT4 recombinants were plated with 0.2 ml of plating cells of a homoimmune lysogen. Small drops of T4 amber mutants at about 10^7 pfu/ml were applied to the lawn of infected cells and the plate incubated overnight at 37^{o}C. Positive responses were indicated by either confluent lysis or plaque formation within the spots.

(ii) 0.2 ml of plating cells of a suppressor-free host was infected with about 10^8 λT4 recombinants, incubated for 15 minutes at 37^{o}C and mixed with 0.2 ml of plating cells of either a λ resistant

or homoimmune lysogenic derivative of the suppressor-free host. The mix was then plated and spots of T4 amber mutants at about 10^7 pfu/ml applied to the lawn. This mixed indicator system allowed some growth of the λT4 recombinants and so increased the sensitivity of the tests. Positive results were indicated as above.

(iii) 0.2 ml of a fresh C600 overnight supplemented with 20 µl of 0.1 M $MgSO_4$ was infected with about 10^8 λT4 recombinants, incubated at 37°C for 10 minutes and then mixed with about 10^8 pfu of a T4 amber mutant. This mix was lysed with a few drops of chloroform after incubating for an hour at 37°C and the lysate serially diluted in phage buffer. Appropriate dilutions were either plated with or spotted onto two λ resistant hosts, one permissive (CR63) and the other non-permissive (W3101/λ) for T4 development.

The first two tests were used to rapidly assay the genetic content of particular λT4 recombinants, whereas the third test was used to quanititate the rescue of a particular T4 marker.

(i) Labelling of polypeptides following infection of UV irradiated cells

An aliquot of an S159 culture was transferred to M9-maltose medium containing 1 mM $MgSO_4$ and grown overnight at 32°C. The culture was diluted next day with fresh medium to an OD_{650nm} of 0.1 on a Unicam spectrophotometer, and grown at 37°C until an OD_{650} of 0.5 was reached. 15 ml of these cells were then pelleted in a bench-top centrifuge, the supernatant discarded and the cells resuspended in 4.5 ml M9-maltose medium containing 20 mM $MgSO_4$, to give a cell concentration of 10^9/ml. The cell suspension was then thinly spread over the surface of a petri dish, irradiated for 15 minutes at 4,500 ergs/mm^2 with a Hanovia Bacteriocidal UV lamp, poured into a foil wrapped bottle to exclude light and so avoid photoreactivation

and left on ice for 10 minutes. 50 µl of bacterial cells were dispensed into small plastic snap-cap tubes, infected with phage at a multiplicity of infection (moi) of 10 and left on ice. After 10 minutes, the tubes were transferred to a 37°C water bath for two minutes and 200 µl of pre-warmed M9-maltose medium containing 0.04 µg/ml of either L-methionine or L-leucine added. 20 µCi of L- ^{35}S methionine or L- ^{14}C leucine was added either three minutes later for labelling polypeptides synthesised at early times or 17 minutes later for those synthesised at late times, and the tubes left at 37°C for 10 minutes. 50 µl of 1 mg/ml L-methionine or L-leucine was then added and the tubes left at 37°C for a further 10 minutes. After this time the tubes were transferred to ice for a few minutes and then spun in a microcentrifuge at 14,500 rpm for six minutes at 14,000 g. The supernatant was then siphoned off, the pellet resuspended in 1 ml ice-cold acetone and the cells pelleted as before. The supernatant was again siphoned off, the pellet resuspended in 50 or 100 µl of sample buffer and the sample placed in a boiling water bath for two minutes. The amount of label incorporated was checked by counting a 1 µl sample in a scintillation counter. Samples were kept frozen at -70°C for up to one month.

(j) SDS polyacrylamide gel electrophoresis of polypeptides

The 25 x 15 cm gels were poured between glass plates separated by 0.15 cm spacers and the edges held by bulldog clamps and sealed with 3% agarose in distilled water. The acrylamide gel solutions were degased after the addition of freshly made Ammonium persulphate and before the addition of TEMED, to remove dissolved oxygen which inhibits the polymerisation process. After the addition of TEMED

the acrylamide gel solution or solutions were either poured directly between the glass plates for linear gels, or into separate chambers of a gradient mixer for gradient gels. Gradient gels were poured by pumping the solution from the gradient maker at a speed of 150 ml/hour into the gel plates. The connection between the chambers of the gradient maker was removed a few seconds after pouring commenced. For all gel types addition of the separating gel was stopped about three to four cm from the top of the gel plates and the surface of the separating gel overlaid with iso-butanol. When polymerisation had occurred (indicated by the appearance of a layer of buffer between the gel and iso-butanol), the iso-butanol and buffer were removed and the gel surface and plates washed with distilled water. The stacking gel acrylamide solution was then de-gased in a vacuum desiccator prior to the addition of TEMED and poured on top of the stacking gel through a pipette. The slot former was then arced into position making sure that no air bubbles were trapped, and the gel left to polymerise. When this had occurred the slot former was carefully removed and the wells cleaned thoroughly with electrophoresis buffer. The bulldog clips and bottom spacer were then removed, the gel plates attached to the electrophoresis tanks with grease and clips and the tanks filled with electrophoresis buffer. Samples, about 5×10^5 counts in 10 to 20 µl, were placed in the wells with a microsyringe. Electrophoresis conditions for gradient gels were at 50 V for about an hour and a half through the stacking gel, and at 80 V for 16 hours through the separating gel, and for linear gels were at 60 V for about 16 hours.

(k) Drying polyacrylamide gels and autoradiography

Polyacrylamide gels of separated proteins were dried on Whatman 3 MM paper under vacuum in a heated Bio-Rad Slab Gel Dryer 224 and placed against a piece of X-ray film for autoradiography. Exposure was usually one to three days depending on the samples used.

(l) Liquid lysates

These were done in three ways depending on the phage used.

(i) λ derivatives

A fresh overnight culture of C600 was diluted 20-fold into L-broth containing 1 mM $MgSO_4$ and grown to OD_{650} 4.5-6.0 (about 2-3 x 10^8 cells/ml), with shaking at 37°C. The cells were then infected with phage at an moi of one and the infected cells kept under the same growth conditions. The OD_{650} of the culture was followed and lysis was judged to have occurred when this value had dropped to its lowest point, usually after two to four hours. Chloroform was then added to lyse the residual cells and the lysate clarified by centrifugation at 10,000 rpm in a 6 x 250 ml rotor in an MSE HS18 centrifuge. The supernatant was then decanted and titred.

(ii) T4 strains for non-glucosylated DNA preparations

A fresh overnight culture of CA7105 was diluted and grown to a cell concentration of 2-3 x 10^8/ml as in part (i). The cells were then infected with the T4 strain at an moi of 10, left standing for 10 minutes and infected again at the same moi in order to induce lysis inhibition. The infected cells were grown at 37°C for two and a half hours, lysed with chloroform and clarified and titred as in part (i).

(iii) T4 strains for cytosine containing DNA preparations

A fresh overnight culture of ED8689 was diluted and grown to a cell concentration of 2-3 x 10^8/ml as in part (i). The cells were then infected at an moi of 0.05 and returned to 37°C. After one cycle of growth enough phage were released to induce lysis inhibition in newly infected cells and the culture was lysed with chloroform after three to four hours. The lysate was clarified and titred as in part (i).

(m) Concentration of phage by polyethylene glycol precipitation

Each litre of unclarified lysate, prepared as in (l) above, was supplemented with 40 g sodium chloride, treated with 1 mg each of DNAase and RNAase and left at room temperature for over one hour. The lysates were then clarified as in (l) above and 10% w/v of polyethylene glycol 6000 (PEG) added to the decanted supernatant. The PEG was dissovled by gentle shaking and the lysate left standing at 4°C overnight. Next day the precipitated phage and cell debris were pelleted at 10,000 rpm in a 6 x 250 rotor in an MSE HS18 centrifuge and the supernatant discarded. The pellets were resuspended in 5-10 mls of phage buffer by gentle shaking at 4°C for about two hours. The lysates were then clarified at 8,000 rpm in a 6 x 250 rotor in an MSE HS18 centrifuge to remove cell debris.

(n) CsCl step-gradients

Gradients were set up in 14 ml centrifuge tubes using steps of 1-1.5 ml each of CsCl solutions of densities 1.3, 1.5 and 1.7 g/ml, made up in phage buffer and clarified in a bench-top centrifuge before use. The least dense solution was added to the tubes first and the denser solutions underlaid using a syringe and 21 gauge hypodermic

needle. The phage sample was then overlaid onto the gradient with a pipette. The gradients were run at 33,000 rpm for two hours in a 6 x 14 MSE Titanium swing-out rotor in an MSE preparative ultra-centrifuge at 20°C. Gradients typically showed three bands, the middle of which contained intact phage and this was collected by side-puncture of the tube with a 25 gauge needle.

(o) Phenol extraction of phage DNA

Phage collected from CsCl step gradients were dialysed, for at least one hour, against 10 mM Tris (pH 7.5), 1 mM EDTA (TE buffer), to remove the caesium and mixed, by gentle shaking, with an equal volume of phenol, that had been equilibrated with an equal volume of 0.5 M Tris (pH 7.5). The layers were then allowed to separate (separation was often accelerated by low-speed centrifugation) and the lower phase, containing phenol, removed. The DNA solution was again mixed with an equal volume of phenol and the extraction repeated three more times. Traces of dissolved phenol were removed by extensive dialysis against TE buffer at 4°C. DNA concentrations were estimated by measuring the absorbance at 260 and 280 nm in quartz cuvettes on a Unicam SP500 UV spectrophotometer.

(p) Restriction of DNA

DNA was digested with restriction endonucleases at 37°C in a total reaction volume of 30 µl containing 10 mM Tris (pH 7.5), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol and 50 mM NaCl except with R.EcoRI where the NaCl concentration was 100 mM and R.SmaI where the NaCl was replaced by 15 mM KCl. The reactions were stopped after the appropriate time, usually one hour, by heating at 70°C for 10 minutes. Reactions were then kept on ice until required.

(q) Ligation of DNA

Restricted DNA, usually at about 20 µg/ml, in a 50 µl reaction volume containing 66 mM Tris (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 10 mM dithiothreitol and 0.1 mM ATP, was mixed with T4 DNA ligase and incubated at 10°C for between three and six hours. The mix was then stored at -8°C and sampled by transfection or transformation.

(r) Transfection

λ recombinant DNA was recovered from ligation reactions, as plaque forming units by the transfection of competent cells. These were made by diluting a fresh overnight of ED8654 (or 802 for ligations involving non-glucosylated T4 DNA) 50-fold into L-broth and growing at 37°C with shaking, until an OD_{650} of 0.4-0.6 had been reached. The cells were then pelleted in a bench-top centrifuge, washed in a half volume of ice-cold 0.1 M $MgCl_2$, resuspended in $^1/_{20}$th the original volume of ice-cold $CaCl_2$ and kept on ice for 30-90 minutes. DNA in 0.1 ml 60 mM $CaCl_2$, 65 mM NaCl, 6.5 mM $Na_3C_6H_5O_7 \cdot 2\ H_2O$, was added to 0.2 ml competent cells and left on ice for 30 minutes. The cells were heat-shocked for two minutes at 42°C and returned to ice for a further 30 minutes. The cells were then plated out on BBL agar plates and incubated overnight at the required temperature for infective centre formation. Transfection efficiencies were usually 0.5-1 x 10^6 pfu/µg intact DNA and recoveries from ligation mixes 1-10 x 10^3 pfu/µg.

(s) Transformation

Plasmid recombinant DNA was recovered in host cells by transformation. This procedure is the same as transfection until the heat shock stage except that HB101/λ and 803/λ cells were commonly

used. After heat shock, 0.7 ml of prewarmed L-broth was added and the mixes incubated for 30-60 minutes at 37°C. 0.2 ml samples were then spread onto L-agar plates supplemented with ampicillin and incubated overnight. Only ampicillin resistant, plasmid containing cells grow on the selective plates.

(t) Preparation of plasmid DNA

Plasmid DNA was prepared in two ways depending on the quantity and purity of DNA required.

(i) Large scale preparations were made by diluting the plasmid bearing strain 50-fold into 100 ml L-broth, containing 40 µg/ml ampicillin, and growing with shaking at 37°C until an OD_{650} of 1.0 had been reached. Chloramphenicol was added to 100 µg/ml to chloramphenicol sensitive strains and the cultures shaken overnight at 37°C. The cells were harvested at 10,000 rpm for five minutes in a 6 x 250 rotor in an MSE HS18 centrifuge, resuspended in 1.5 ml ice-cold 25% sucrose in 0.05 M Tris (pH 8.0) and swirled gently on ice for five minutes. 0.5 ml of fresh lysozyme (10 mg/ml) in sucrose solution was added and, swirling continued for another five minutes. 0.5 ml of ice-cold 0.5 M EDTA (pH 8.0) was then added, the mixture left on ice for five minutes and 2.5 ml triton mix added. (Triton mix = 0.1% triton X-100, 60 mM EDTA (pH 8.0), 50 mM Tris (pH 8.0).) After swirling for 10 minutes on ice, the mixture was clarified at 15,000 rpm for 30 minutes in a 8 x 50 rotor in an MSE HS18 centrifuge. The non-viscous supernatant was decanted into a measuring cylinder and 1 g of CsCl and 0.05 ml 10 mg/ml ethidium bromide added for each ml of supernatant. The mixture was poured into 14 ml centrifuge tubes, topped up with liquid paraffin and spun for 48-72 hours at 35,000 rpm in a 6 x 14 MSE Titanium swing-out

rotor in an MSE preparative ultracentrifuge at 20°C. The resultant gradients showed two bands, an upper chromosomal and a lower plasmid band. The plasmid band was collected by side puncture of the tube with a 25 gauge needle and passed through a Dowex (50 W - X8 from BDH) column with TE buffer to remove the ethidium bromide and caesium. The plasmid DNA was then precipitated with two to three volumes of ethanol at -20°C and pelleted at 10,000 rpm for 30 minutes in an 8 x 50 MSE rotor in an MSE HS18 centrifuge at 0°C. The supernatant was discarded and the pellet dried in a vacuum desiccator and resuspended in 200 µl TE buffer. The DNA concentration was estimated as in (o).

(ii) Small scale plasmid DNA preparations for rapidly screening putative recombinants were made by pelleting 0.5 ml of a fresh overnight of the plasmid bearing strain in L-broth containing 40 µg/ml ampicillin, in a snap cap tube in a microcentrifuge. The cells were resuspended in 100 µl of an ice-cold lysis solution containing 25 m<u>M</u> Tris (pH 8), 10 m<u>M</u> EDTA (pH 8), 50 m<u>M</u> glucose and 2 mg/ml lysozyme, and left on ice for 30 minutes. 200 µl of 1% SDS in 0.2 <u>M</u> NaOH was then added and the mix left on ice for five minutes. 150 µl of 3 <u>M</u> sodium acetate (pH 4.8) containing 300 µg/ml carrier tRNA was added and the suspension left on ice for 60 minutes with occasional mixing. The preparation was centrifuged for five minutes at room temperature in a microcentrifuge and 400 µl of the supernatant removed, mixed with 1.0 ml of ethanol and left at -20°C for 30 minutes. The precipitate was pelleted in a microcentrifuge and the supernatant discarded. The pellet was redissolved in 100 µl 0.1 <u>M</u> sodium acetate (pH 6) mixed with 200 µl of ethanol and left at -20°C for 10 minutes. The plasmid DNA was pelleted in a microcentrifuge and dissolved in

75 µl restriction endonuclease buffer. The final DNA concentration was typically about 40 µg/ml.

(u) Electrophoresis of DNA fragments

DNA fragments generated by restriction endonuclease digestion were separated by electrophoresis through either 1% agarose or 10% polyacrylamide gels.

(i) Agarose gel electrophoresis

1.5 g of agarose was dissolved in 150 ml of electrophoresis buffer (0.04 M Tris, 0.02 M sodium acetate, 1 mM EDTA (pH8.2)) by boiling, poured onto a 13 x 24 cm horizontal gel apparatus and left to set. The slot former was then removed and the gel slab connected to the wells by two thicknesses of "Jay Cloth" soaked in electrophoresis buffer. 40 µl samples in loading buffer (10% PEG, 0.025% bromophenol blue, 0.05% Xylene cyanol FF and 0.05% Orange G) were pipetted into the wells and run into the gels at about 3 volts/cm. When the samples had been run into the gel, the wells were topped up with electrophoresis buffer and the gel covered with "cling-wrap". Electrophoresis was then at about 25 volts across the gel for 16-17 hours.

(ii) Polyacrylamide gel electrophoresis

These gels were made by mixing 25 ml of acrylamide solution, 3 ml freshly made 1.6% Ammonium persulphate . 42 ml 0.09 M Tris-borate (pH 8.3) and 2.5 mM EDTA and degasing the solution in a vacuum desiccator. 25 µl TEMED was then added to trigger polymerisation and the mix poured between 15 x 15 cm glass gel plates, whose sides and bottom edge had been sealed with 3% agarose and held in place with bulldog clips. The slot former was then arced into position to avoid trapping air bubbles. After polymerisation had occurred

the bottom spacer, slot former and bulldog clips were removed and the plates fixed between the tanks with grease and bulldog clips. The slots were thoroughly cleaned and the samples applied in loading buffer. Electrophoresis was at 40 V for about 16 hours.

(iii) Staining of gels and photography

Both types of gels were stained in 2 mg/l ethidium bromide for 30 minutes and destained in distilled water for a further 30 minutes. Gels were photographed under UV light by a seven minute exposure through an X4 red filter using Ilford FP4 film.

(v) Nick translation

DNA for hybridisation probes were labelled in vitro by nick translation. 10 µCi ^{32}P α-dCTP was dried down in a vacuum desiccator and resuspended in 20 µl of 50 mM Tris (pH 7.5), 5 mM $MgCl_2$ containing 1 µg of the appropriate DNA, 5 µg/ml bovine serum albumen and 15 µM dATP, dGTP and dTTP. 1 µl of DNAase I and ½ µl DNA polymerase I were added and the reaction incubated at 15°C for two hours. The reaction was stopped with 200 µl of 50 mM NaCl, 10 mM Tris (pH 8), 1 mM EDTA (TNE buffer) and the percentage incorporation of label estimated by washing 5 µl from the reaction, spotted onto a 2.1 cm disc of grade 3 Whatman paper, in 5% trichloroacetic acid, and counting the label retained on the filter in a couple of mls of scintillation fluid in a scintillation counter. The enzymes in the reaction were denatured with a few drops of chloroform and a drop of Orange G marker dye added. The mix was then applied to the top of a 15 x 0.7 cm Sephadex G-50 column and eluted with TNE buffer. 0.5 ml fractions were collected and elution stopped when the Orange-G had reached the bottom of the column (Orange-G marks the position of unincorporated nucleotides). The fractions were then

monitored by Cerenkov counting on a scintillation counter and the peak fractions (usually 3-5) pooled and stored at -8°C.

(w) Transfer of DNA to nitrocellulose filters

DNA was transferred from agarose gels, plaques and bacterial colonies as follows:

(i) Southern transfer

DNA fragments in agarose gels were denatured in 0.5 M NaOH, 1.5 M NaCl for 45 minutes, and neutralised in 1 M Tris (pH 5.5), 3 M NaCl for 45 minutes. Treated gels were placed on two thicknesses of photoprint paper, soaked in 20 X SSC and supported on a glass plate, ensuring that no air was trapped between the gel and paper (1 X SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). The edges of the paper dipped into a reservoir of 20 X SSC so that they acted as wicks. An exact gel sized piece of nitrocellulose soaked in 2 X SSC was placed on top of the gel, again ensuring that no air was trapped between gel and filter. A similar sized piece of photoprint paper soaked in 2 X SSC was placed on top of the nitrocellulose filter and a stack of same sized paper, about one inch thick placed on top of this. The whole apparatus was covered in "cling wrap" and weights placed on top of the paper stack. Overnight, capillary action drew the SSC from the reservoir, through the gel and nitrocellulose filter. Denatured DNA carried with the SSC bound to the filter, which was washed in 2 X SSC, dried and baked for two hours at 80°C in a vacuum oven to strengthen the binding, next day.

(ii) Tranfer of DNA from plaques

A circular piece of nitrocellulose, just smaller than a petri-dish, was placed on the surface of BBL-agar plate with a top layer containing a bacterial lawn and plaques, and left for a few minutes.

The filter was then peeled off and placed plaque-side up on a filter paper pad soaked in 0.5 M NaOH for 10 minutes, to denature the DNA bound to the filter. The filter was neutralised on a series of three filter paper pads soaked in 0.5 M NaOH/1.5 M NaCl, 1.5 M NaCl/0.5 M Tris (pH 7.5) and 0.2 M Tris (pH 7.5)/2 X SSC respectively for five minutes each. The filters were then dried and baked in a vacuum oven at 80°C.

(iii) Transfer of plasmid DNA from bacterial colonies

Colonies of plasmid-containing bacteria were 'toothpicked' into an ordered array on an L-agar plate containing ampicillin and a replica made on a nitrocellulose filter placed on top of a similar plate. After overnight incubation at 37°C, the filter was peeled off and placed on a filter paper pad soaked in 0.5 M NaOH for 10 minutes. The filter was subsequently treated as in part (ii) above.

Phage showing positive hybridisation subsequently can be recovered from the original plaque that its DNA was transferred from and plasmid DNA showing similar hybridisation recovered from the appropriate colony on the replica plate.

(x) Hybridisation conditions for DNA on nitrocellulose filters and autoradiography

About 5 x 10^5 cpm of nick translated DNA probe was transferred to a snap-cap tube, denatured at 90°C for 10 minutes and transferred to 25 ml of hybridisation mix consisting of 50% formamide, 0.03 M Tris (pH 7), 0.75 M NaCl, 1 mM EDTA and 0.2% SDS. The mix was poured into a polythene bag containing the filters to be probed and the bag sealed with a Calor "Easy seal" bag sealer, making sure that all air had been expelled. The bag was then incubated at 37°C with shaking for 16-48 hours. The filters were removed from the bag and

washed in a large volume (over 1 litre)of 4 X SSC at room temperature for 45 minutes, a similar volume of 4 X SSC at 65°C for 45 minutes and finally the same volume of 2 X SSC for 30 minutes at room temperature. The filter was then attached to a piece of photo-print paper and dried. The paper was labelled with radioactive ink and attached to a lead backed folder. A sheet of flash-sensitised X-ray film was then placed on top of the filter and a fast tungstate intensification screen placed on top of this. The folder was then shut and another lead back folder placed on top. The whole construction was then sealed inside a black polythene bag to exclude any light and left at -70°C for 16-72 hours before developing.

(y) Isolation of DNA from agarose gels

100 µg of DNA was digested with restriction endonucleases and loaded into a single 10 cm slot in a 1% agarose gel. After the gel had been run, the edges of the gel were cut off and stained and destained with ethidium bromide. The pieces were then repositioned on the gel and the gel viewed under UV light. The position of the band to be isolated was marked on the stained gel slices and the corresponding section of the rest of the gel cut out. The slice of agarose was fragmented by forcing it through a 10 ml syringe and dissolved in 25 ml of a saturated Potassium iodide solution at room temperature. About 100 mg of Bio-Rad DNA grade Hydroxyapetite (HAP) that had been equilibrated with saturated Potassium iodide solution, was added and left to adsorb the DNA for about 10-20 minutes. The HAP was then pelleted by low-speed centrifugation, washed and repelleted three times in saturated Potassium iodide to remove remaining agarose and finally resuspended in 10 mM Na_2PO_4, 1 mM EDTA (pH 6.9). The HAP was washed several times in this solution by

sedimentation and resuspension and poured into a small column made from a pasteur pipette. This was washed with two to three column volumes of the same solution and the DNA eluted with one to two volumes of 0.4 M Na_2PO_4, 1 mM EDTA (pH 6.9). The eluate was clarified by low-speed centrifugation to remove traces of HAP and dialysed first against 1 M NaCl, 10 mM Tris (pH 8) for three hours with two buffer changes and then against TE buffer overnight. Two to three volumes of ethanol were added to the solution next day, and the DNA precipitated at -20°C for two hours. The DNA was pelleted at 40,000 rpm in an MSE 6 x 14 Titanium swing-out rotor in an MSE preparative ultracentrifuge for 30 minutes at 0°C, the supernatant decanted and the pellet dried in a vacuum desiccator. The pellet was resuspended in 100 µl TE buffer and the amount of DNA estimated by running 10 µl of the preparation on a 1% agarose gel.

Figure 3.1

Turbid and clear plaque forming λ*td*$^{+}$ recombinants.

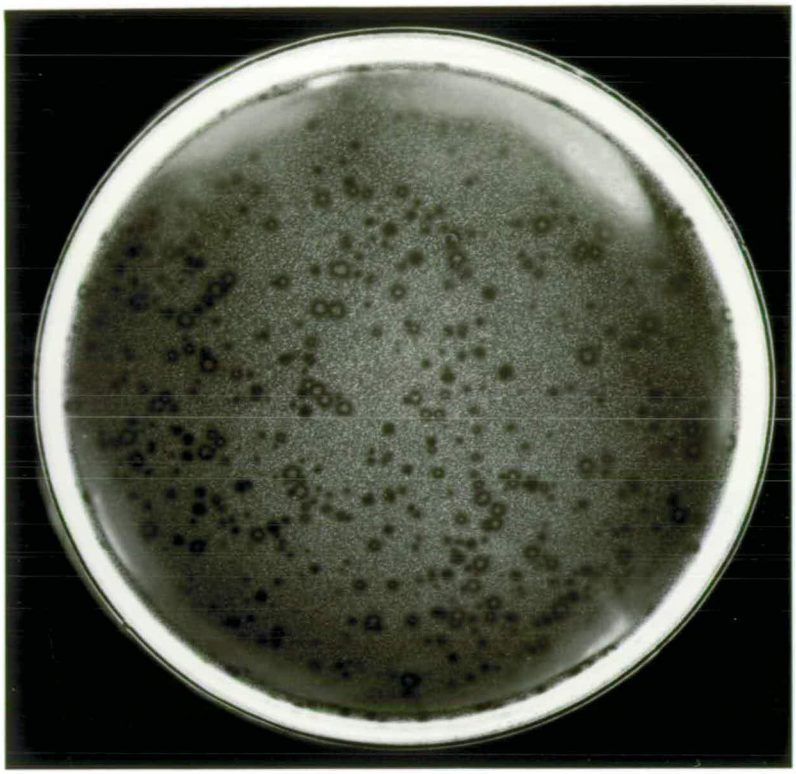


Table 3.1 Frequency and plaque morphology of λ*td* recombinants derived from different T4 DNAs

λ vector	T4 DNA used in generation of recombinants	Restriction endonuclease used in generation of recombinants	Frequency of λ*td*$^+$ recombinants in pooled lysates of λT4 phages	plaque morphology[1] of λ*td* recombinants
816	*alc*4	R.*Eco*RI	2-5%	t, c
540	*alc*1	R.*Hind*III	1%	c
616	*denA*	R.*Eco*RI	0.1%	c

Notes: 1. c = clear t = turbid

In the case of λ*td* recombinants generated from R.*Eco*RI digested *alc*4 DNA, turbid plaque forming derivatives occurred about 50 times more frequently than clear plaque forming derivatives.

Table 3.2 Characteristics of λtd recombinants

Name of λtd	source of T4 DNA	λ vector or λtd derived from	T4 DNA fragments included in kb	orientation of T4 DNA insert
-1	alc4	816	2.7 EcoRI	l
-4	alc4	816	2.7;0.7 EcoRI	l
-10	alc1	540	5.3;1.0 HindIII	r
-10Δ	alc1	λtd-10	1.0 HindIII	r
-18	alc1	540	5.3 HindIII	r
-30	alc1	540	5.3;1.0 HindIII	l
-30Δ5	alc1	λtd-30	5.3 HindIII	l
-30Δ1	alc1	λtd-30	1.0 HindIII	l
-31	alc1	540	5.3;1.0 HindIII	r
-563	alc4	616	2.7;0.7 EcoRI	l
-611	denA	616	2.7;0.7;3.0 EcoRI	r
-652	denA	616	2.7;0.7;3.0 EcoRI	l
-611T	denA	616	2.7;0.7;3.0 EcoRI	r

Notes:

1. Thy = growth in the absence of thymine: + = growth, - = no growth

2. TM = growth in the presence of trimethoprim: R = growth, S = no growth

3. + = lysogens formed, - = lysogens not formed

4. + = polypeptide synthesised by λtd derivative

na = not applicable nt = not tested

plaque characteristics			lysogen	characteristics		T4 polypeptides synthesised[4]				
morphology	Thy[1]	TM[2]	formed[3]	Thy[1]	TM[2]	nrdA	td	X	frd	Y
turbid	+	S	+	+	S		+			+
turbid	+	S	+	+	S		nt			
clear	+	R	-	na	na	+	+		+	+
turbid	-	R	+	-	R				+	
clear	-	S	-	na	na	+	+			+
clear	+	R	-	na	na	+	+		+	+
clear	-	S	-	na	na	+	+			+
turbid	-	R	+	-	R			nt		
clear	+	R	-	na	na	+	+		+	+
turbid	+	S	-	+	S		+			+
clear	+	S	-	na	na	+	+	+		+
clear	+	S	-	na	na	+	+	+		+
turbid	+	S	+	+	S			nt		

3. RESULTS

1. Organisation and expression of the thymidylate synthetase region of the T4 genome.

a) Isolation of λtd$^+$ recombinants

Pooled lysates of λT4 recombinants derived from ligation mixes involving various λ and T4 strains and either R.EcoRI or R.HindIII (see Tables 3.1 and 3.2) were examined for the presence of λtd$^+$ recombinants by looking for plaque-forming units on the thyA host ED8614 in the absence of thymine. λtd$^+$ recombinants formed normal sized plaques under these conditions whereas other T4 recombinants and λ vectors either formed tiny plaques or failed to plate under the same conditions (Figure 3.1). The presence of limiting amounts of thymine in the plating mix, allowed the formation of a weak lawn and made plaque visualisation easier. λtd$^+$ recombinants were isolated at a frequency of between 0.1 and 5 per cent amongst phage in pooled lysates, depending upon the donor DNA and restriction endonuclease used in their generation (see Table 3.1). These differences in frequency of generation were later found to be correlated with physical structure. The origin and properties of relevant λtd recombinants and their derivatives are listed in Table 3.2.

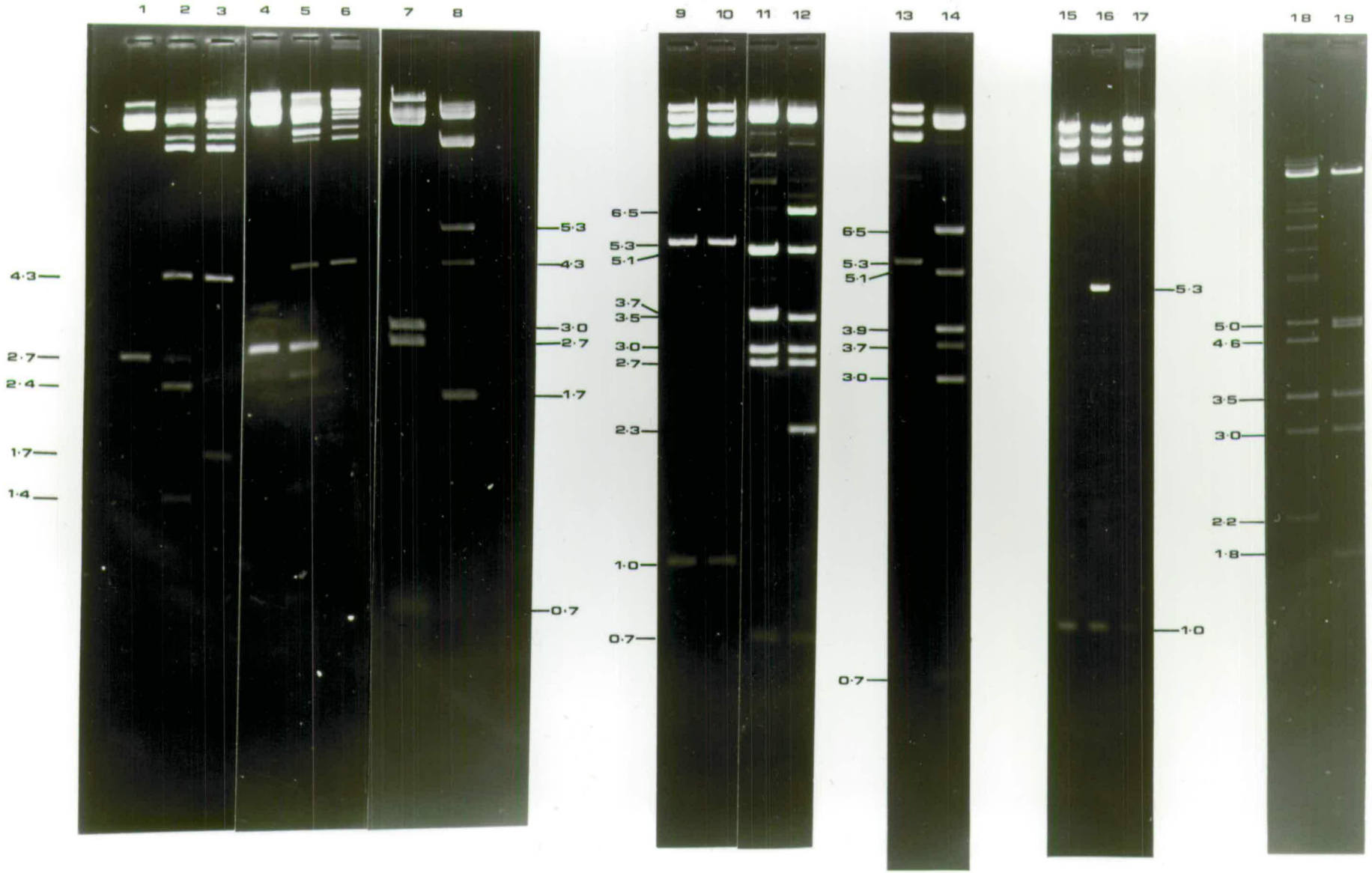
b) Structural characterisation of λtd$^+$ recombinants and their derivatives

The DNA content of many λtd$^+$ recombinants was analysed by agarose gel electrophoresis after restriction endonuclease digestion (Fig. 3.2). λtd$^+$ recombinants generated by R.EcoRI all possessed at least a 2.7 kb EcoRI fragment and all those generated by R.HindIII contained two Hind II fragments of 1.0 and 5.3 kb. These fragments were shown to be derived from T4 DNA as they gave positive hybridisation to a

Figure 3.2

Structural analysis of the DNA of λtd recombinants. Tracks 1-3 contain λtd-1 DNA; 4-6, λtd-4 DNA; 9 and 11, λtd-30; 7 and 8, λtd-652; 10, 12 and 16, λtd-31; 13 and 14, λtd-18; 15 and 18, λtd-10Δ; 17 and 19, λtd-30Δ1. The DNA in tracks 1, 4, 7, 11, 12, 14, 18 and 19 was digested with R.EcoRI; that in tracks 3, 6, 8, 9, 10, 13, 15, 16 and 17 by R.HindIII; and that in tracks 2 and 5 by a combination of the two enzymes. The figure represents portions of different ethidium bromide stained, 1% agarose gels. Estimates of fragment sizes in kb are indicated.

Figure 3·2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5·1
3·9
3·7
3·0
0·7
5·3
1·0
5·0
4·6
3·5
3·0
2·2
1·8

laballed probe made from T4 DNA. Turbid plaque forming λtd$^+$ recombinants generated by R.EcoRI frequently carried an additional EcoRI fragment of 0.7 kb and all clear plaque forming derivatives generated similarly, contained a 3.0 kb EcoRI fragment in addition to those of 2.7 and 0.7 kb.

Double digestion of the DNA of λtd$^+$ recombinants by R.EcoRI and R.HindIII elucidated the structural organisation of the region: the 2.7 kb EcoRI fragment contains an R.HindIII site 0.3 kb from one end; the R.HindIII generated λtd$^+$ recombinants contained the 2.7 and 0.7 kb EcoRI fragments; R.EcoRI generated recombinants carrying all three EcoRI fragments, contained the 5.3 kb HindIII fragment and yielded double digest products of 0.3 and 0.8 kb.

It was thought that derivatives of the λtd$^+$ recombinants generated by R.HindIII, carrying only one of the HindIII fragments would be unable to complement the thyA host, since all such λtd$^+$ recombinants, carried both HindIII fragments. In order to test this hypothesis, the 0.7 kb EcoRI fragment, which is contained within the 5.3 kb HindIII fragment, was transferred to pBR325 and used to make a labelled probe. This probe could identify the presence of the 5.3 kb HindIII fragment in plaque hybridisation experiments against pooled lysates of λT4 recombinants generated by R.HindIII previously shown not to contain λtd$^+$ derivatives. This was to eliminate the presence of λtd$^+$ phages which would hybridise to the plasmid probe and so necessitate screening for recombinants carrying only the 5.3 kb HindIII fragment. One plaque gave positive hybridisation in this test and the phage isolated from it could not complement the thyA host. Examination of the DNA of this phage, λtd-18, revealed that it carried the 5.3 kb HindIII fragment alone

and included the 0.7 kb EcoRI fragment. A similar derivative, λtd-30Δ5, has been isolated following in vitro deletion, the sequential action of R.HindIII and T4 DNA ligase on λtd-30. Other in vitro deletion experiments have lead to the isolation of td-10Δ and -30Δ1 which only carry the 1.0 kb HindIII fragments and were derived from λtd-10 and -30 respectively. All derivatives carrying only one of the HindIII fragments do not complement the thyA host and are designated λtd⁻.

A labelled probe made from λtd-4 DNA when hybridised against a nitrocellulose filter carrying T4 DNA digested by various restriction endonucleases after transfer from an agarose gel, showed positive hybridisation to bands of 1.0, 1.9 and 5.3 kb in R.HindIII digests of T4 DNA (Figure 3.3). A similarly derived nitrocellulose filter was hybridised against a labelled probe made from λtd-10Δ DNA and showed positive hybridisation to bands of 2.7 and 4.2 kb in R.EcoRI digests of T4 DNA

(Figure 3.3). The 4.2 kb EcoRI fragment, identified by the λtd-10Δ probe, is the same size as an EcoRI fragment of T4 DNA known to carry g 32 (Selzer et al, 1978). The 1.9 kb HindIII fragment showing positive hybridisation to the λtd-4 probe, was faint and represented a partial digest product, implying that the 1.0 kb HindIII fragment is contiguous with another HindIII fragment of 0.9 kb. The data presented in this and the proceding two paragraphs, unambiguously established the structural organisation of the td region of the T4 genome, shown in Figure 3.4.

The T4 DNA of λtd recombinants is inserted into the λ genome in both possible orientations. Phages λtd-1, -4, -652 and -30 are representative of phages carrying their T4 DNA inserts in one

Figure 3.3

Identification of DNA fragments in the td region of the T4 genome using hybridisation probes. A: ethidium bromide stained 1% agarose gel; B: autoradiograph of a nitrocellulose filter derived from this gel, and C: track from ethidium bromide stained 1% agarose gel and autoradiograph of a nitrocellulose filter derived from it after hybridisation to the labelled probes. For A, track 1 contains DNA from λtd-1; 2 from λtd-4; 3 from λtd-5; 4 from λtd-7; 5 from λtd-8; 6 from λNM816; 7 from λNM886; 8-11 from T4 alc7. The DNA in C is from T4 JW819. DNA in tracks A.1-7 and 10 and C.1 is digested with R.EcoRI; A.8 by R.BglII; A.9 by R.XhoI; A.11 by R.HindIII. The labelled probe used in B was made from λtd-4 DNA and that used in C from λtd-10Δ DNA. Estimates of fragment sizes in kb are indicated.

N.B. λtd-5 carries the 2.7 and 0.7 kb EcoRI fragments from the td region plus two others from different regions; λtd-7 and -8 are similar to λtd-1; λNM886 carries the E.coli thyA gene.

Figure 3·3

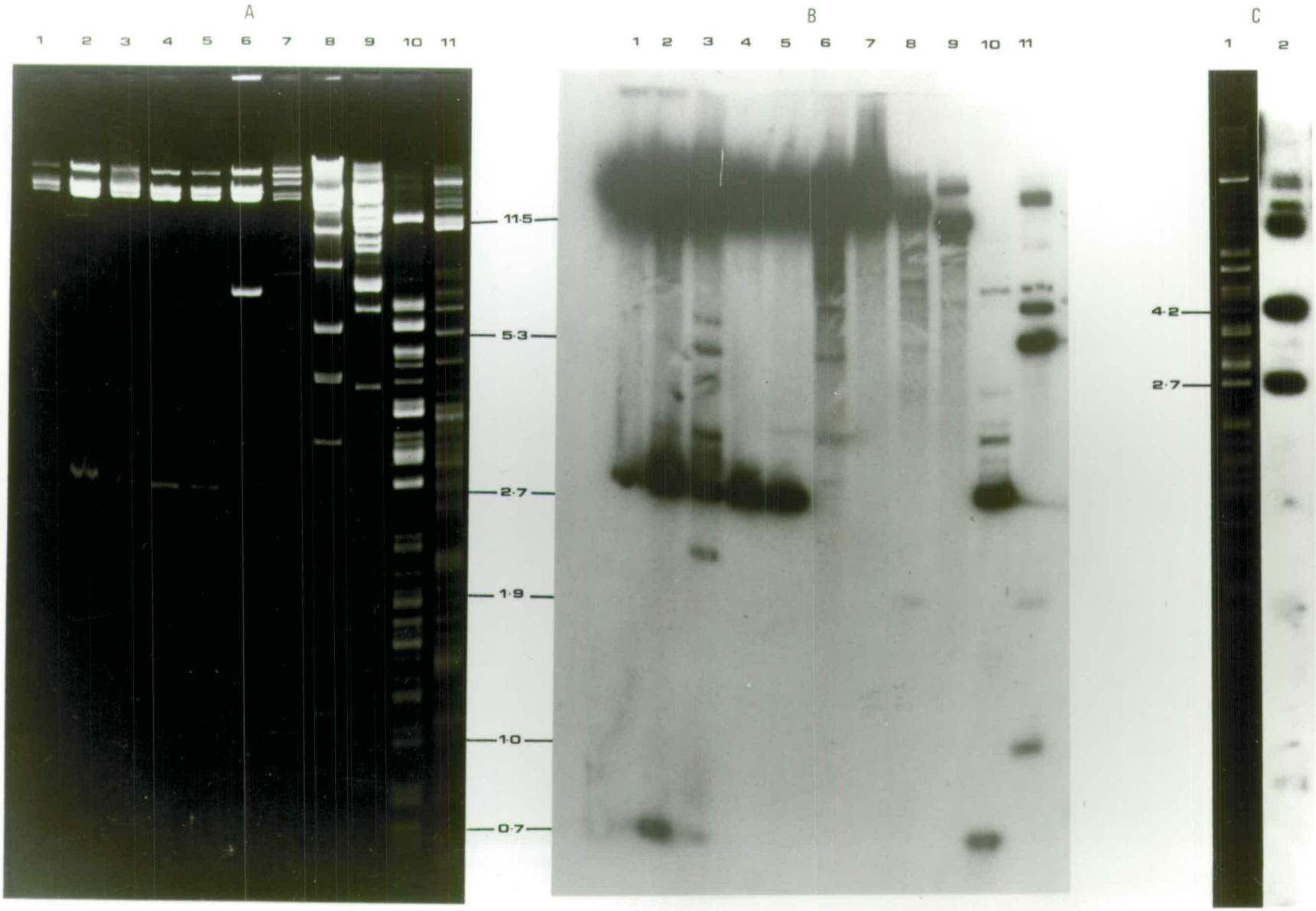
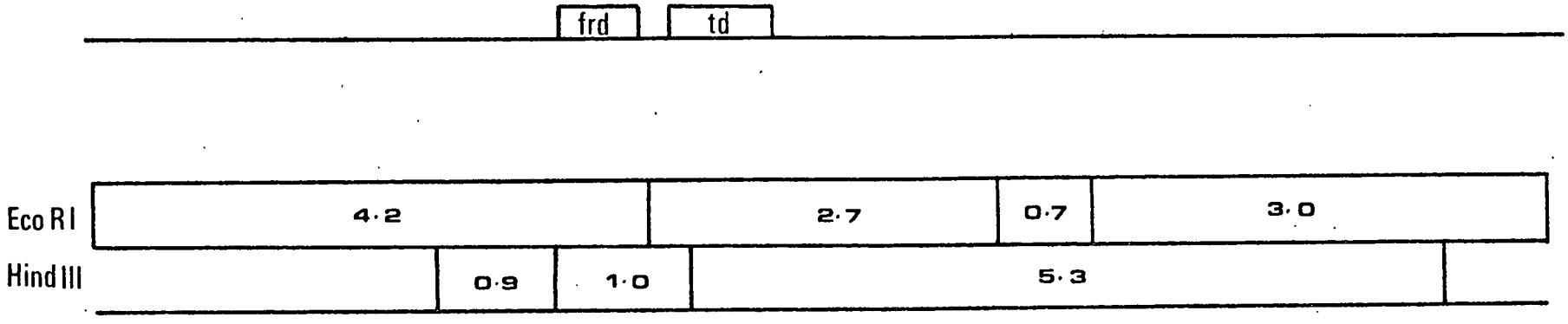


Figure 3.4

Physical map of the td region of the T4 genome. The rectangles represent the minimum sizes of the frd and td genes (Wood and Revel, 1976). The fragment sizes are in kb.

Figure 3.4



orientation, while λtd-611, -10, -31 and -18 of the opposite orientation. The properties of the λtd$^+$ phages and their derivatives are listed in Table 3.2.

c) Genetic characterisation of λtd$^+$ recombinants and their derivatives

The T4 td gene must lie within the 2.7 kb EcoRI fragment, as this is the only fragment common to all λtd$^+$ recombinants. λtd derivatives carrying only the 5.3 kb HindIII fragment, such as λtd-18 and -30Δ5, or only the 1.0 kb HindIII fragment, such as λtd-10Δ and -30Δ1, do not complement the thyA host and so are td$^-$. This indicates that the R.HindIII site within the 2.7 kb EcoRI fragment lies inside the coding sequence of the td gene.

Having determined the position of the td gene within the physical map of the region, the λtd$^+$ recombinants and their derivatives were tested for the presence of the neighbouring frd gene which is the structural gene for dihydrofolate reductase. Both the T4 and E.coli reductases, are inhibited by the folate analogue trimethoprim (Mathews, 1969; Breeze et al, 1975). T4 mutants that overproduce this enzyme can be identified by their ability to confer resistance to folate analogues (Chase and Hall, 1975). Thus it was hoped that λT4 recombinants carrying the frd gene could induce the synthesis of dihydrofolate reductase in host cells and raise the concentration of this enzyme well above the level of that in uninfected cells. This increase in enzyme concentration would allow the phage to produce plaques on an E.coli host at concentrations of trimethoprim inhibitory to that host.

λtd$^+$ recombinants generated by R.EcoRI, failed to produce plaques on ED8689 at inhibitory levels of trimethoprim, but those generated

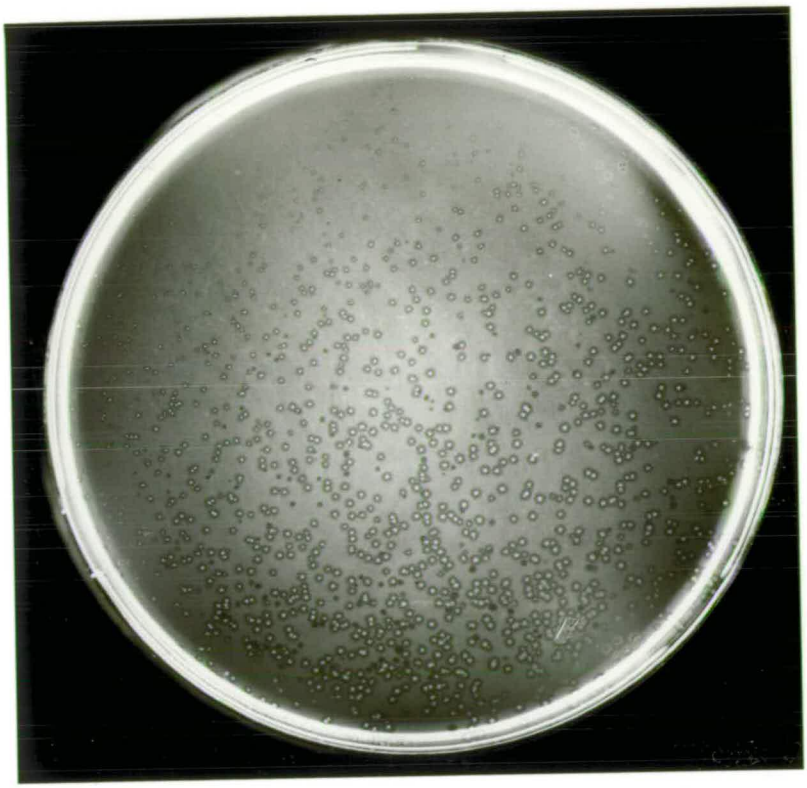
by R.HindIII formed clear plaques under the same conditions (Figure 3.5). Deletion derivatives of R.HindIII generated recombinants behaved differently. Those containing only the 5.3 kb HindIII fragment, λtd-18 and -30Δ5, failed to produce plaques under selective conditions, whereas those carrying only the 1.0 kb HindIII fragment, td-10Δ and -30Δ1, formed large turbid plaques under the same conditions. These data indicate that the frd gene lies within the 1.0 kb HindIII fragment.

The localisation of the td and frd genes within the physical map allows the alignment of the physical and genetic maps of the region (see Figure 3.4) and indicates that the 4.2 kb EcoRI fragment which hybridises to the λtd-10Δ probe (see part (b) above) is the same as that identified by Selzer et al (1978) as carrying g 32.

Pooled lysates of λT4 recombinants generated by either R.EcoRI or R.HindIII were investigated for the presence of λfrd$^+$ phage, and while none was found in lysates generated by R.EcoRI, they were common in those generated by R.HindIII. λfrd$^+$ recombinants generated by R.EcoRI would have to include at least the 4.2 kb EcoRI fragment that carries g 32. Recombinants carrying a functional g 32 have never been isolated, but H. Krisch (pers. comm.) has isolated the g 32 coding sequence on a HindIII fragment derived from the T4 DNA carrying an amber mutation in g 32. Host cells carrying this fragment inserted into pBR322 grow 40% slower than similar cells containing the plasmid vector due to weak suppression of the g 32 amber mutation by the host. Studies involving sub-fragments derived from the original plasmid showed that g 32 was responsible for this deleterious effect. Indeed another gene coding for a DNA binding protein, ipI, has interfered with the cloning of the T4 tRNA genes (Fukada et al, 1980b).

Figure 3.5

Turbid and clear plaque forming λ*frd*$^{+}$ recombinants.



All λtd phages have been analysed for the presence of g 63 by marker rescue tests, using two g 63 amber mutants, but with negative results.

In order to test for the presence of T4 promoters that could serve the frd and td genes, lysogenic derivatives for λtd$^+$ and λfrd$^+$ phages were made using thyA and trimethoprim sensitive hosts respectively. Lysogens of λtd$^+$ phages, representative of both possible orientations of the T4 DNA insert (λtd-1, -4 and the turbid derivative of λtd-611; see Table 3.2), were thymine independent, and those of λfrd$^+$ phages, representative of both possible orientations of the T4 DNA insert (λtd-10Δ and λtd-30Δ1), were trimethoprim resistant. As the main λ promotors are repressed in the lysogenic state, the 1.0 kb HindIII and 2.7 kb EcoRI fragments seem to include T4 sequences that act as promotors for the frd and td genes respectively. Cured derivatives of these lysogens were either thymine requiring or trimethoprim sensitive.

Work involving complementation studies or the measurement of enzyme levels in lysogens for λ derivatives carrying genes lacking a known promotor, has suggested the existance of weak rightwards and leftwards constitutive promotors in the b2 region of the λ chromosome. Hopkins et al (1976) noted that a trpA host lysogenic for a λ recombinant carrying the trpA gene in the l orientation (trpA here is transcribed from the P_L promotor) was auxotrophic, whereas a similar host lysogenic for a phage with the trpA gene in the r orientation (trpA here is transcribed from P'_R), grew slowly in the absence of tryptophan and readily segregated prototrophic derivatives. The authors suggested that this behaviour could be explained by the presence of a weak, constitutive rightwards promotor

in the b2 region of the λ genome. The λ vector used in the generation of these λtrp phages was NM540, so it is possible that the expression of gp frd in lysogens of λtd-10Δ or -30Δ1, is from the same promotor. However trimethoprim resistance is usually accompanied by at least a two fold increase in the level of dihydrofolate reductase (Chase and Hall, 1975; Breeze et al, 1975), so it seems unlikely that expression of the frd gene in lysogens for λtd-10Δ or -30Δ1, is from such a weak promotor. Indeed, as gp frd is expressed in strains lysogenic for λfrd derivatives carrying the 1.0 kb HindIII fragment in both possible orientations, it is most likely that a T4 promotor is included on this fragment.

Ptashne (1978) described the synthesis of β-galactosidase from a hybrid operon in which transcription of the lacZ gene was from the prm promotor of λ. A DNA fragment carrying this hybrid operon was carried in b_2 region of a λimm^{21} phage so that both the P_L and prm promotors initiated transcription in the same direction. The binding of repressor to prm, at low repressor concentrations, is necessary for transcription of the cI gene that it serves, and so the synthesis of β-galactosidase, from the hybrid operon, was not expected in the absence of repressor. However, a low level of β-galactosidase was consistently synthesised from the hybrid operon in the absence of repressor. This finding could be explained by the presence of a weak constitutive leftwards promotor between att and the srIλ2 site in the b2 region of the λ chromosome. D. Burt (pers. comm.) has analysed control signals on the λ chromosome by fusing λ DNA fragments to the lacZ gene or inserting λ DNA fragments between the trp promotor and the lacZ gene in a fused operon. In the former case, expression of the lacZ gene indicates the presence of a promotor on the λ DNA fragment, whereas the non-expression of

the lacZ gene in the latter case, indicates the presence of a transcription termination signal on the λ DNA fragment. This type of analysis has shown that there is no promotor between att and the shnλ3 site on the λ chromosome and so if the low level expression of lacZ in the absence of λ repressor from the fused operon described by Ptashne (1978), is indeed from a weak constitutive λ promotor, it must lie between the srIλ2 and shnλ3 sites. It is possible that the expression of the td gene in lysogens involving λtd phages of one of the two possible orientations, is from such a promotor, but since thyA lysogens involving λtd phages of both possible orientations are thymine independent, it seems that the 2.7 kb EcoRI fragment carries a T4 promotor that can initiate the transcription of the td gene.

The clear-plaque phenotype of λtd recombinants carrying either the 3.0 kb EcoRI or 5.3 kb HindIII fragments, seems to indicate the presence of a function that interferes with either the establishment, or maintenence of lysogeny, within the 2.2 kb common to these two fragments (see Figure 3.4). Furthermore, while the 2.7 and 0.7 kb EcoRI fragments can be readily transferred to pBR325, several attempts to transfer the 3.0 kb EcoRI fragment, to the same plasmid, have failed. Again the implication is that the 3.0 kb EcoRI fragment codes for a product that is deleterious to the host. This cannot involve the denA product, endonuclease II, as the clear plaque phenotype occurs irrespective of the denA genotype of the donor DNA. Turbid derivatives of these clear plaque forming λtd recombinants could help identify the T4 function that blocks the establishment or maintenence of lysogeny, especially those carrying an amber mutation in this function. Such derivatives have been isolated, e.g. a

turbid derivative of λtd-611, but attempts to isolate turbid derivatives that could be restored to the clear plaque phenotype in the presence of an amber suppressor have failed. The function involved could be identified as a band that is present after electrophoresis of ^{35}S labelled polypeptides derived from UV-irradiated cells infected with a clear plaque forming λtd phage, but absent from those derived from infections by a turbid derivative.

A correlation between plaque size and orientation of the T4 DNA insert of λtd$^+$ recombinants was noticed, under selective conditions, such that phage carrying the td region in one orientation formed larger plaques than those carrying the same insert in the opposite orientation. This observation is consistent with early expression of the td gene from the λ P_L promotor in recombinants of the former orientation and with late expression from the P'_R promotor in recombinants of the latter orientation.

d) Proteins synthesised by λtd derivatives

The examination of labelled polypeptides following SDS-polyacrylamide gel electrophoresis of proteins derived from UV-irradiated cells, infected with λtd derivatives, has revealed the presence of five new polypeptides. These are identified as T4 specific as they are only induced following infection with recombinant phage and their synthesis is dependent on the orientation of the T4 DNA insert. They appear at early times after infection by the group of λtd phages which form large plaques under selective conditions, now designated l orientation phages, but only at late times after infection by the group of λtd phages which formed small plaques under selective conditions, now designated r orientation phages (see introduction for details of the time of appearance of products transcribed from P_L or P'_R).

R.EcoRI generated recombinants carrying all three EcoRI fragments, such as λtd-652, specify four T4 polypeptides, and those generated by R.HindIII carrying the two HindIII fragments, such as λtd-30, also specify four T4 polypeptides, three of which are identical to those specified by the R.EcoRI generated recombinants. The labelled polypeptides derived from λtd-30 and -652 infections were separated on SDS polyacrylamide gels, along with those derived from infections involving T4 mutants, defective in functions encoded by the T4 td region, with a view to identifying the polypeptides specified by the λtd recombinants (Figure 3.6).

The largest polypeptide induced by both λtd-30 and -652 is identified as gp nrdA and corresponds to a band missing in T4 del (63-32)1 (Figure 3.6, lanes e and o), a deletion shown to be defective in gp nrdA activity (Homyk and Weil, 1974). The apparent Mr of this polypeptide is about 82.5000, a value in good agreement with that of 85,000 determined for gp nrdA by Berglund (1975). The nrdA polypeptide is also missing in T4 del (63-32)9, where it seems to be replaced by a slower moving, fusion polypeptide of apparent Mr 85,000 (Figure 3.6, lane b). T4 del (63-32)9 has also been shown to lack gp nrdA activity (Homyk and Weil, 1974). gp nrdA is not induced by either λtd-1, -563, or -10Δ (see Figure 3.7), indicating that the nrdA gene is wholly included, or at least partly extends into, the 3.0 kb EcoRI fragment.

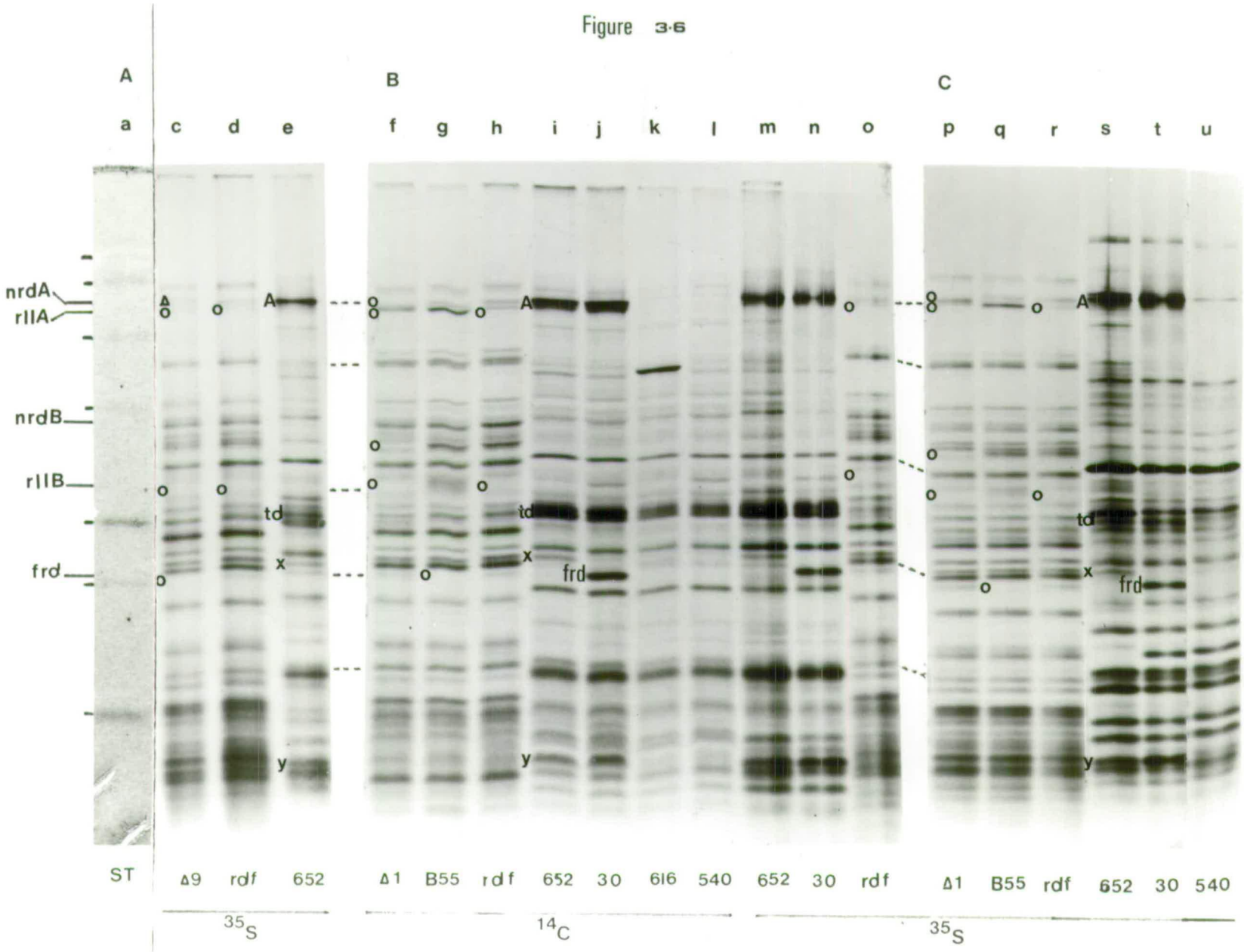
The next largest polypeptide is also induced by both λtd-30 and -652 and has an apparent Mr of 28,000. This polypeptide migrates in a crowded region of the gel, which increases the difficulty in scoring its absence from certain λtd derivatives and T4 mutants. However in all of many experiments, the intensity of a band of the

Figure 3.6

Autoradiographic analysis of ^{35}S and ^{14}C labelled polypeptides after electrophoresis through a 10-20% gradient SDS polyacrylamide gel. T4 mutants were labelled from 3-7' and λ derivatives from 3-13', except tracks r-t, where labelling was 17-27'. Track a contains standard proteins stained with Coomassie blue: β-galactosidase (128,000); phosphorylase a (92,000); bovine serum albumen (68,000); ovalbumen (42,000); penicillinase (28,000); myokinase (21,000); egg lysozyme (14,000); and cytochrome c (12,000). These standards correspond to the unlabelled lines to the right of track a. Tracks c, d, f, g, h, o, p, q and r are T4 mutants; k, l and u are λ vectors, and e, i, j, m, n, s and t are λtd recombinants.

Δ1 = del (63-32) 1; Δ9 = del (63-32) 9; B55 = amB55; rdf - rEDdf41; o = missing bands, Δ = an alteration of the nrdA polypeptide in Δ9; and A, td, x, frd and y are bands present in λtd recombinants, but not λ vectors.

Figure 3·6


A
B
C
a
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u
nrdA
rIIA
nrdB
rIIB
frd
ST
Δ9
rdf
652
Δ1
B55
rdf
652
30
616
540
652
30
rdf
Δ1
B55
rdf
652
30
540
35S
14C
35S

appropriate mobility is greater for those phages identified as td$^+$, than for their td$^-$ derivatives (Figure 3.7, lanes r and w versus t and x). This band was best resolved when the polypeptides, produced after late infections of UV-irradiated cells, by λtd$^+$ and λtd$^-$ derivatives, were run on a linear 10% SDS polyacrylamide gel (Figure 3.7, lane y versus z). Although T4 del (63-32)1 and 9 should not show this band, as they have been shown to be deficient in gp td activity (Homyk and Weil, 1974), the crowding in the appropriate region of the gel, makes this observation impossible. The apparent Mr of 28,000 is in good agreement with a value of 29,000 determined by Capco et al (1973).

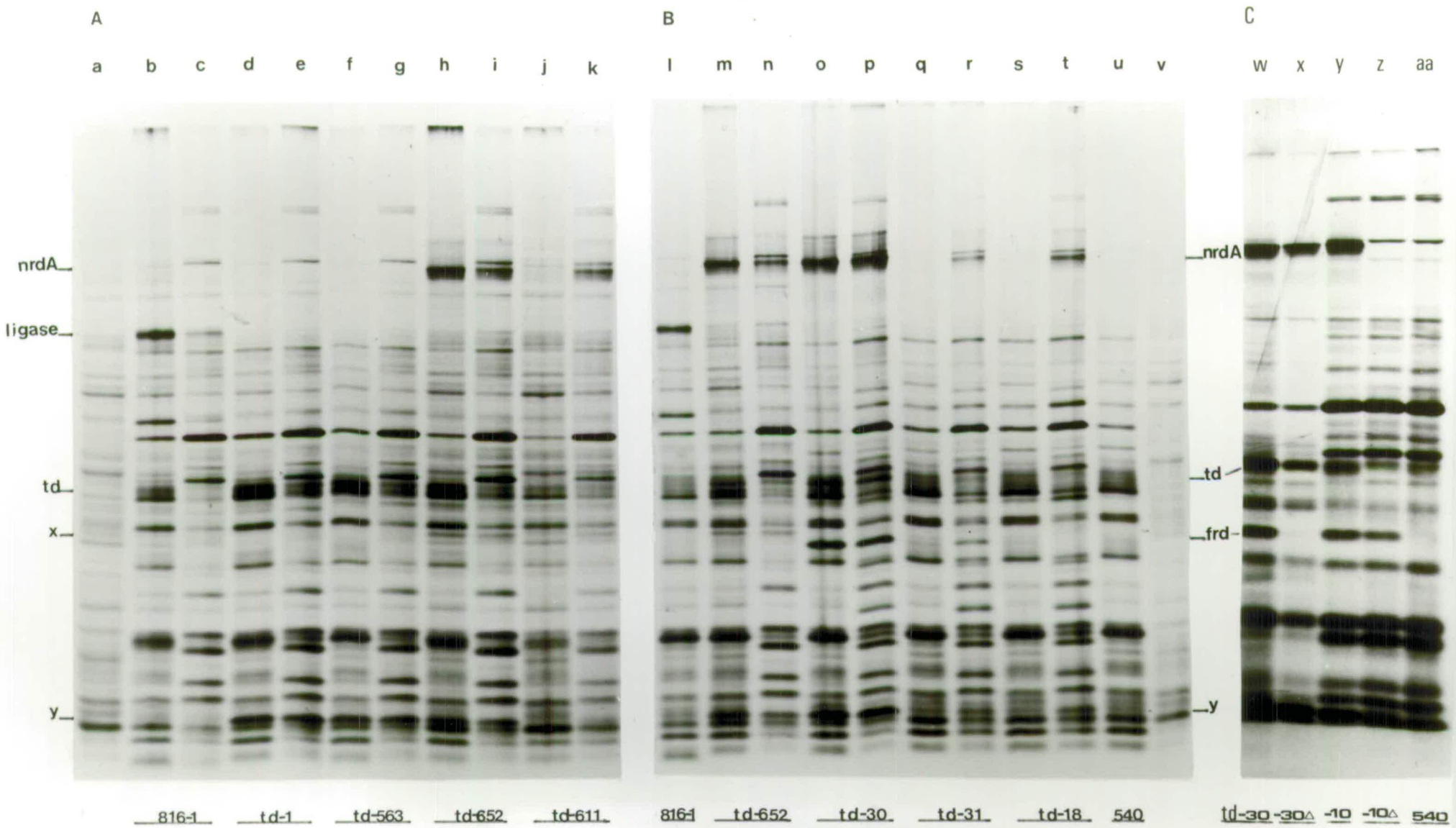
A polypeptide of apparent Mr 24,000 is only induced by λtd derivatives carrying all three EcoRI fragments, such as λtd-652 (Figure 3.7) and so must be wholly, or partly, confined to the 0.8 kb region of the 3.0 kb EcoRI fragment that is not included in the 5.3 kb HindIII fragment (see Figure 3.4). Assuming that the molecular weight of an average amino acid is 120, then 0.8 kb of DNA could code for a polypeptide of about 27,500 and so this polypeptide, designated x, could be specified by this 0.8 kb region. The nature of polypeptide x is unknown, but the position of its coding sequence suggests that it could be gp denA. However neither the molecular weight of gp denA, nor the nature of the denA mutation used in this work, is known.

The fourth polypeptide has an apparent Mr of 22,500 and is only induced by R.HindIII generated λtd$^+$ recombinants, such as λtd-30 and -31, or the λtd$^-$ derivatives carrying only the 1.0 kb HindIII fragment, such as λtd-10Δ (Figure 3.7, lanes y and z not x). This polypeptide is missing from T4 del (63-32)9 and amB55 (Figure 3.6, lanes b, f and p), both known to lack gp frd activity, but present in all

Figure 3.7

Autoradiographic analysis of polypeptides specified by λtd derivatives labelled with ^{35}S, at early (3-13') and late (17-27') times after infection. A and B were 10-20% gradient, and C a 10% linear, SDS polyacrylamide gel. Tracks a and v are uninfected cells labelled early; u and aa are 540 infections labelled early and late respectively; a, b, d, f, h, j, l, o, q, s, u, v, w and x were λtd infections, labelled early; and c, e, g, i, k, n, p, r, t, y, z and aa were λtd infections, labelled late.

Figure 3.7


A
a b c d e f g h i j k
nrdA
ligase
td
x
y
816-1 td-1 td-563 td-652 td-611
B
l m n o p q r s t u v
816-1 td-652 td-30 td-31 td-18 540
C
w x y z aa
nrdA
td
frd
y
td-30 -30△ -10 -10△ 540

other T4 controls. The apparent Mr of this polypeptide agrees well with a value of 22,000 recently reported by Mosher and Mathews (1979), using antibody to detect the enzyme. These data unambiguously identify this polypeptide as the product of the frd gene.

The fifth polypeptide, apparent Mr 11,300, is induced by all λtd$^+$ phages and their derivatives, except those that only carry the 1.0 kb HindIII fragment such as λtd-10Δ (see Figure 3.7). The function of this polypeptide is unknown and is here designated y. Polypeptide y must be specified by the 2.4 kb segment of the 2.7 kb EcoRI fragment common to all recombinants that induce its synthesis (see Figure 3.4). This means that the coding sequence for polypeptide y must lie between the td and nrdA genes and represents a previously unknown T4 gene.

It was expected that a polypeptide corresponding to gp nrdB, apparent Mr 35,000 (Berglund, 1975), would be specified by those λtd recombinants that specified gp nrdA. However a polypeptide of this size was not seen amongst either ^{35}S, or ^{14}C, labelled polypeptides, induced in λtd phage infections. A comparison of the physical map of the td region and the end points of the T4 deletions in the region (see Figure 3.15), predicts that T4 del (63-32)1, which inactivates both nrdA,B and denA activities (Homyk and Weil, 1974), does not extend as far towards g 63 as the T4 DNA content of λtd phages carrying the 3.0 kb EcoRI or 5.3 kb HindIII fragments. Thus such λtd recombinants should carry the nrdB gene, making the failure to detect gp nrdB a puzzling feature of this analysis. B.-M. Sjoberg (pers. comm.) has in fact shown that a Q$^-$, S$^-$ derivative of λtd-611 is as good a source of the B subunit of T4 ribonucleotidediphosphate reductase (gp nrdB) as T4 itself. The T4 DNA insert in this phage

is in the r orientation, but cannot be transcribed from P'_R in the derivative studied, due to the absence of a functional Q gene product. Thus it seems likely that a T4 promotor able to serve the nrdB gene is carried on the T4 DNA insert of this phage.

e) Direction of transcription

The experiments described above demonstrated that the T4 specified polypeptides were made at early times in infection, if the T4 DNA insert was in the l orientation, such as in λtd-30 and -652 (Figure 3.7, lanes h, m and o), but only at late times if the same DNA insert was in the r orientation, such as λtd-31 and -611 (Figure 3.7, lanes k and r). This means that the transcription of the T4 DNA inserts of λtd-30 and -652 is initiated from the λ P_L promotor and that of λtd-31 and -611 from the λ P'R promotor (see Wilson and Murray, 1979), implying that the td region is normally transcribed from the frd gene towards g 63, that is anti-clockwise on the T4 map.

2. Organisation of the T4 genome between the td and DNA ligase genes

Heteroduplex mapping of T4 deletion mutants has established the extent of the td-DNA ligase region of the genome (Homyk and Weil, 1974; G.G. Wilson, pers. comm.; see Figure 3.15). The leftwards limits of del (63-32)1 and 10 approximates to the left-hand end of the td gene, since these deletion mutants lack gp td activity but retain gp frd activity (Homyk and Weil, 1974). Two overlapping deletions, Δ30.1 and Δ61, define the approximate rightwards limit of g 30, as the former lacks, but the latter possesses, DNA ligase activity (G.G. Wilson, pers. comm.). Thus the left-hand end of Δ61 defines the right-hand limit of g 30. These data limit the extent of the entire td-g 30 region to a size of 18 kb. However since the

1.9 kb HindIII fragment contains g 30 intact (Wilson and Murray, 1979), and the λ recombinants carrying the 5.3 kb HindIII fragment are td⁻, the maximum size of the remainder of the region between the left-hand end of the 1.9 kb and the right-hand end of the 5.3 kb HindIII fragments is about 11 kb.

a) A single HindIII fragment covers the region between td and DNA ligase

Although many different λT4 recombinants have been isolated (see for example Wilson et al, 1977; Velten and Abelson, 1980), marker rescue tests have failed to detect recombinants carrying either g 31 or g 63, suggesting that either these genes or adjacent genes carried on the same restriction fragments, code for functions that are deleterious to E.coli or λ development. These negative results prompted the use of λtd and λlig recombinants as probes to investigate the structural organisation of the td-DNA ligase region of the T4 genome, by identifying overlapping fragments.

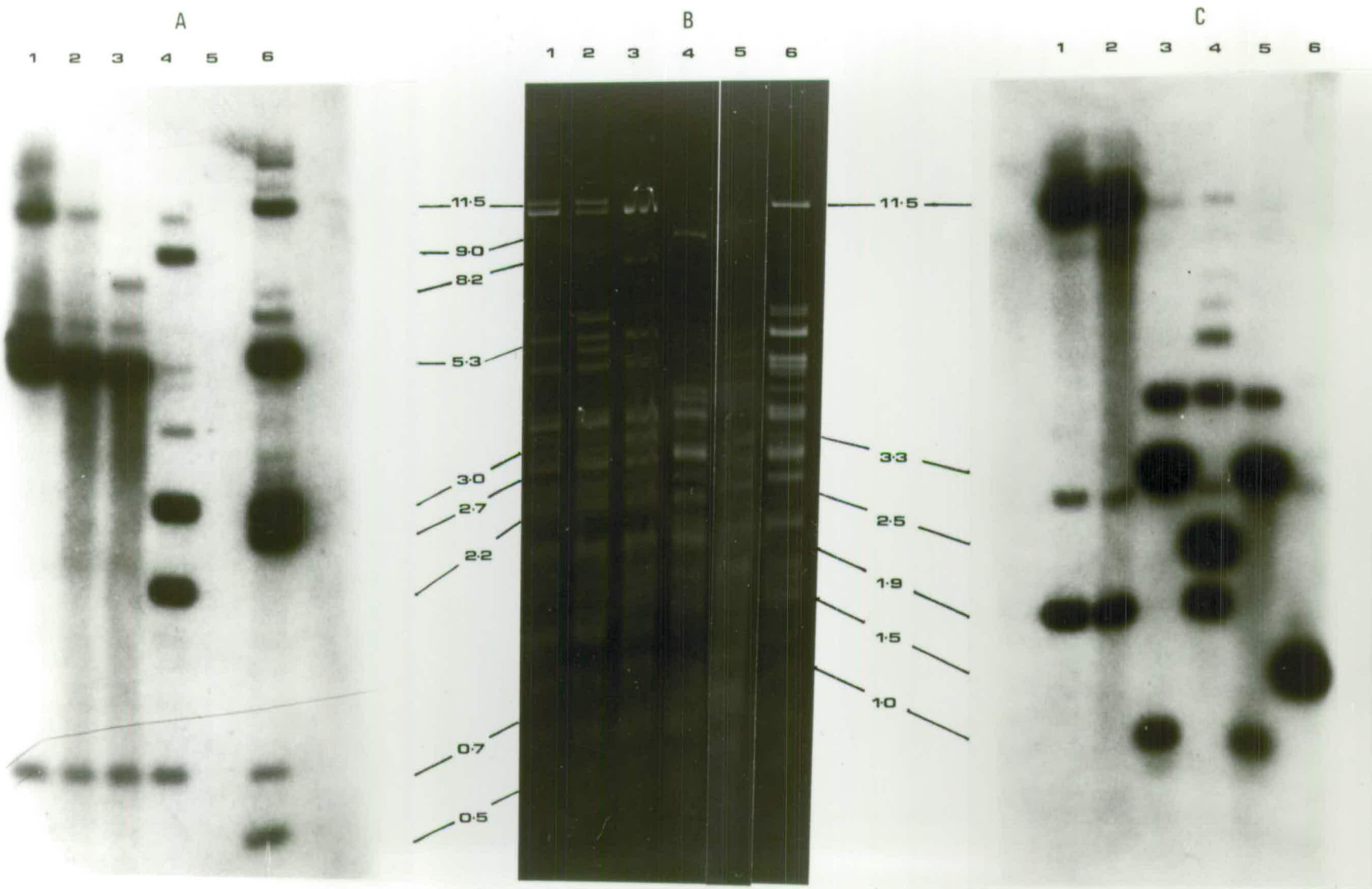
As the 3.0 kb EcoRI fragment carried in λtd phages, and the 1.5 kb EcoRI fragment carried in some λlig phages, extend further into the td-DNA ligase region than any known HindIII fragments, probes made from λT4 recombinants carrying these EcoRI fragments, were hybridised against R.HindIII digests of the T4 DNA transferred to nitrocellulose filters. In addition to showing positive hybridisation to the previously identified overlapping HindIII fragments, both probes showed positive hybridisation to a T4 DNA HindIII fragment of about 11.5 kb (Figure 3.8). As this value is the same as the estimate for the size of the td-DNA ligase region from heteroduplex studies, it seemed almost certain that this region is spanned by a single HindIII fragment of 11.5 kb.

Three bands of approximately 11 kb, a doublet and a slightly

Figure 3.8

Identification of DNA fragments in the td-DNA ligase region using hybridisation probes. B: ethidium bromide stained 1% agarose gel; A and C: autoradiograph of nitrocellulose filters derived from this and an identical gel after hybridisation with the labelled probes. Tracks 1-6 are digests of T4 JW819 DNA using (1) R.HindIII; (2) R.HindIII and R.BamHI; (3) R.HindIII and R.BglII; (4) R.HindIII and R.PstI; (5) R.HindIII, R.BamHI and R.BglII; (6) R.EcoRI. The labelled probe used in A was made from λtd-652 DNA and that used in C from λlig6-2 DNA. Track A.5 contains no DNA and A.6 is a mixture of T4 JW819 DNA digested with R.EcoRI and R.HindIII, R.BamHI and R.BglII. Estimates of fragment sizes are indicated.

Figure 3·8


A
1 2 3 4 5 6
B
1 2 3 4 5 6
11·5
9·0
8·2
5·3
3·0
2·7
2·2
0·7
0·5
11·5
3·3
2·5
1·9
1·5
1·0
C
1 2 3 4 5 6

slower moving single band, are seen after electrophoresis of R.HindIII digested T4 DNA, through agarose gels (see Figure 3.8). In order to identify which of these bands is derived from the td-DNA ligase region, T4 DNA was digested with R.HindIII in conjunction with other restriction enconucleases that only cut T4 DNA a limited number of times (Figure 3.8). Digestion with R.HindIII and R.BamHI removed one fragment from the doublet, R.HindIII and R.BglII removed the single band and R.HindIII and R.PstI removed all three bands. These digests when transferred to nitrocellulose filters and hybridised to λtd and λlig probes, unambiguously identified the upper band of 11.5 kb, as the fragment comprising the td-DNA ligase region (Figure 3.8). These data also show that the 11.5 kb HindIII fragment contains an internal BglII fragment of 8.2 kb and yields a 9 kb HindIII/PstI derivative, which both hybridise to the λtd, but not the λlig, probes.

b) Attempt to clone the 11.5 kb HindIII fragment

Since previous attempts to clone the 11.5 kb HindIII fragment from R.HindIII digests of whole T4 DNA had failed, it was decided that the chances of recovering this fragment would be improved by using DNA isolated from the 11 kb region of an agarose gel after electrophoresis of R.HindIII digested T4 DNA as a source of donor DNA. DNA isolated in this way was inserted into λNM762. It was hoped that any deleterious functions carried by this fragment would be tolerated in this vector, which only grows lytically, or that there would be derivative fragments, deficient in the deleterious function, amongst the population of DNA fragments recovered from agarose gels.

The whole 11 kb region was removed from agarose gels and so contained all three HindIII fragments of this approximatete size.

No attempt was made to purify the fragment carrying the td-DNA ligase region as one of the other two HindIII fragments was known to carry genes 6-12 (Mileham, Revel and Murray, unpublished observations) and contain the unique R.BamHI site of T4 DNA. This fragment provided a useful internal control for the attempt to clone the HindIII fragment carrying the td-DNA ligase region.

Plaques recovered after transfection from the ligation mix, were screened for the loss of supF and positive hybridisation against a labelled probe made from T4 DNA. 67 such recombinants were further screened for positive hybridisation against a labelled probe made from the DNA of a pBR325 derivative, containing the 1.5 kb EcoRI fragment that overlaps the 11.5 kb HindIII fragment at the g 30 side. None of these recombinants showed positive hybridisation to the plasmid probe, but all 67 showed marker rescue for T4 amber mutants in genes 6-12 but not in g 31 or g 63.

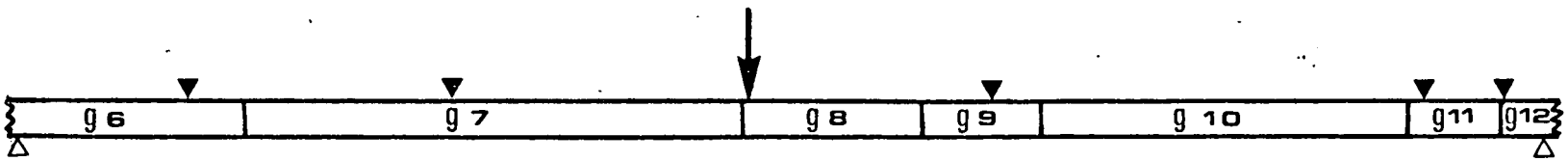
The DNA of several of these recombinants was examined by restriction endonuclease analysis. All carried a 10.5 kb HindIII fragment containing an internal R.BamHI site and internal EcoRI fragments of 3.7, 3.1, 1.8 and 0.5 kb, which had been previously identified within the g 6-12 region of the T4 genome (Wilson et al, 1977). A map of the g 6-12 region is shown in Figure 3.9. The R.BamHI site was within the 3.7 kb EcoRI fragment, a finding in agreement with other work (V. Tanyashin, pers. comm.; Wilson et al, 1980).

The failure to recover recombinants carrying the 11.5 kb HindIII fragment containing the td-DNA ligase region, must be due to deleterious functions specified by the fragment, as the 10.5 kb HindIII fragment carrying genes 6-12 was repeatedly recovered. The same must be true of the other member of the 10.5 kb HindIII doublet, as recombinants carrying this fragment were not isolated.

Figure 3.9

Physical map of the g 6-g 12 region of the T4 genome. The rectangles represent the minimum sizes of these genes, ▼, R.EcoRI targets, Δ, R.HindIII targets and the downward pointing arrow, the R.BamHI target. The fragment sizes are in kb.

Figure. 3·9



The procedure was repeated using pBR322 as a vector and 46 transformants, from this ligation mix, were found to be tetracycline sensitive and ampicillin resistant. 36 of these derivatives scored positive in a colony hybridisation test against a labelled probe made from T4 DNA. All 36 of these derivatives showed marker rescue for T4 amber mutants in genes 6-12 but not in g 31 or g 63.

c) Identification and cloning of the EcoRI fragments from the td-DNA ligase region

A consideration of the genetics of the region suggested that the failure to recover recombinants carrying the 11.5 kb HindIII fragment, might have been due to the presence of g 31 or the rIII gene (see strategy section of the Introduction). Both of these genes lie to the g 30 end of the 11.5 kb HindIII/and so it was thought that the fragment use of a derivative fragment, lacking this region, could obviate this problem. The 9 kb HindIII/PstI fragment (see Figure 3.8), was chosen for further analysis as it lacks about 2.5 kb of DNA from the g 30 end of the 11.5 kb HindIII fragment. This fragment was isolated from a preparative agarose gel and shown to be essentially free from other DNA fragments by gel analysis (Figure 3.10).

A labelled probe made from this purified DNA fragment, was used in a hybridisation test against a complete R.EcoRI digest of T4 DNA transferred to a nitrocellulose filter, in order to identify internal and overlapping EcoRI fragments (Figure 3.11). Five bands were detected as giving positive hybridisation, and these had sizes of 1.1, 1.3, 1.7, 2.2 and 3.0 kb. The intensity and shape of the slowest moving band suggested a readily explained doublet; one fragment (3.0 kb) was that previously isolated within λtd phages such as λtd-611 (see Figure 3.11) and the second fragment (3.1 kb), was

Figure 3.10

Isolation of the 9 kb HindIII/PstI fragment. Track 1 contains T4 JW819 DNA digested with R.EcoRI; track 2, T4 JW819 DNA digested with R.HindIII and R.PstI; track 3, isolated 9 kb HindIII/PstI fragment; track 4, T4 JW819 DNA digested with R.HindIII. Estimates of fragment sizes are indicated.

Figure 3·10

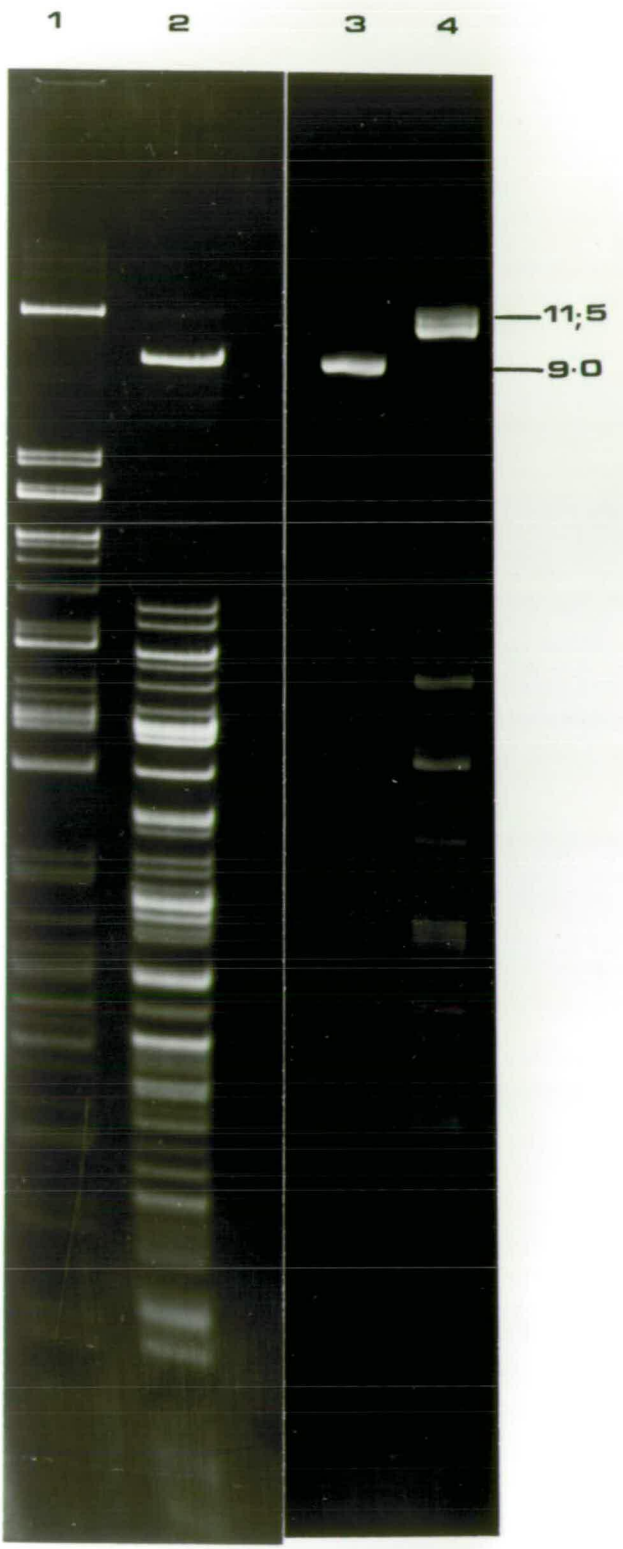
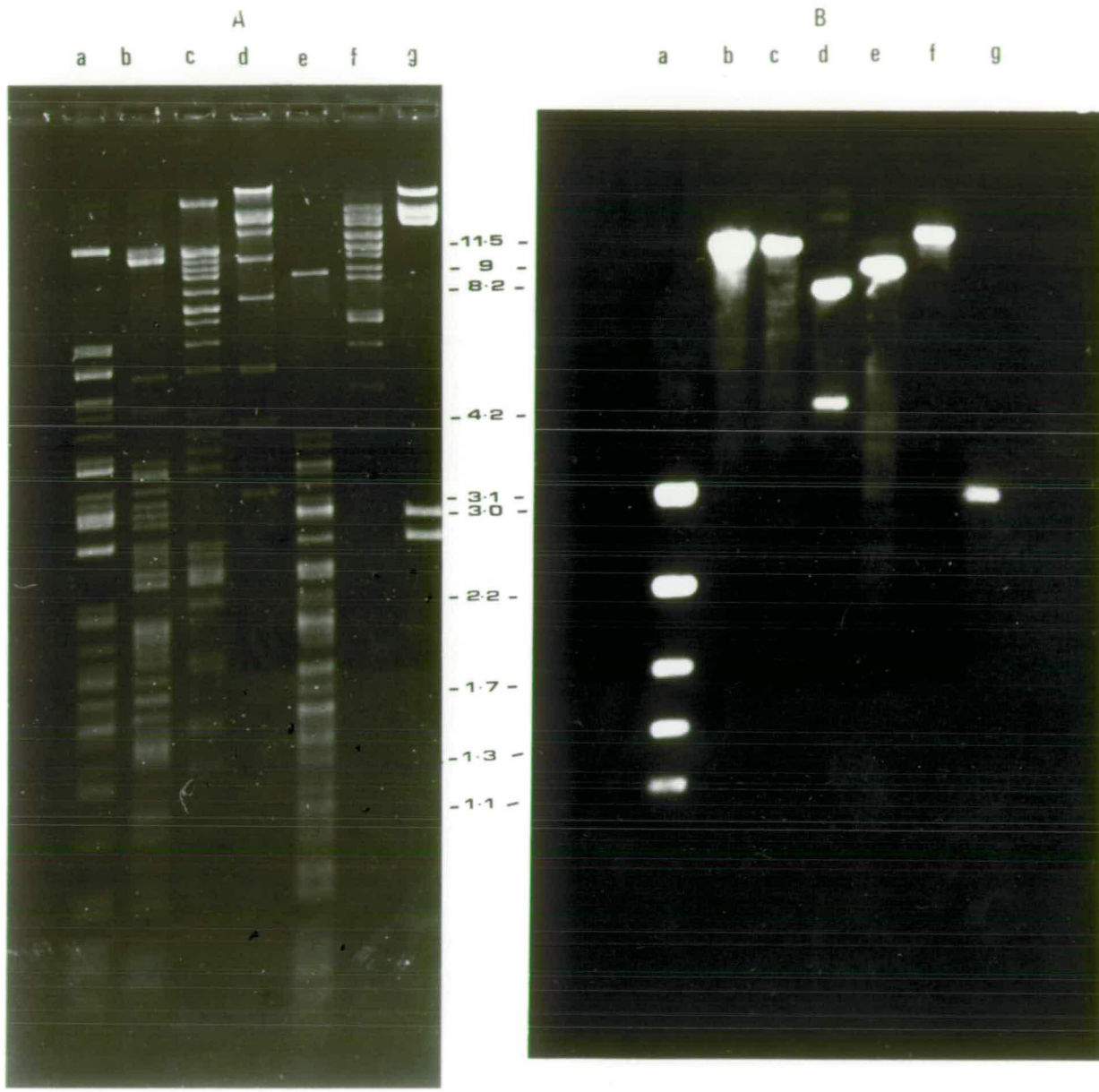


Figure 3.11

Identification of DNA fragments that hybridise to the ^{32}P-labelled 9 kb, HindIII/PstI fragment probe. A: ethidium bromide stained 1% agarose gel; B: autoradiograph of nitrocellulose filter, derived from this gel, after hybridisation to the labelled probe. Estimates of fragment sizes in kilobase pairs are shown in the centre. Tracks a-f are digests of T4 JW819 DNA using (a) R.EcoRI; (b) R.HindIII; (c) R.PstI; (d) R.BglII; (e) R.HindIII and R.PstI; (f) R.XhoI. Track (g) : λtd-611 DNA digested with R.EcoRI.


A
a b c d e f g
-11·5 -
- 9 -
- 8·2 -
- 4·2 -
- 3·1 -
- 3·0 -
- 2·2 -
- 1·7 -
- 1·3 -
- 1·1 -
B
a b c d e f g

that anticipated from the g 30 end of the 11.5 kb HindIII fragment (this fragment may be less than 3.1 kb, but this designation differentiates it from the 3.0 kb EcoRI fragment). Evidence for the existence and position of the 3.1 kb EcoRI fragment was obtained when a labelled probe made from the DNA of a pBR325 derivative, carrying the 1.5 kb EcoRI fragment from the g 30 region, strongly hybridised to a band of 4.5 kb in a partial R.EcoRI digest of T4 DNA (Figure 3.12). This identification was later confirmed following the cloning of a 2.7 kb deletion derivative of the 3.1 kb EcoRI fragment. Thus the 11.5 kb HindIII fragment overlaps an EcoRI fragment of approximately 3 kb at each end.

Isolated 9 kb HindIII/PstI fragment was also digested with R.EcoRI and the resultant fragments inserted into λNM607. This vector was chosen for two reasons; putative recombinants could be rapidly screened by the insertional inactivation of the cI gene, and cloned functions may be poorly expressed, at least in the r orientation, so reducing their possible deleterious effects. 42 derivatives of clear plaque morphology, scored positive in a plaque hybridisation test against a labelled probe made from T4 DNA. DNA was prepared from all of these recombinants and investigated by restriction endonuclease analysis. 41 derivatives carried single EcoRI fragments of the following sizes: 0.12, 1.1, 1.7, 2.2 and 2.7 kb (see Figure 3.13). Labelled probes were made from the DNA of representatives of each class of recombinant and hybridised against T4 DNA, digested with various restriction endonucleases, on nitrocellulose filters. All five classes gave diagnostic hybridisation patterns confirming that they originated from the td-DNA ligase region (Figure 3.14). A single recombinant, containing a 0.5 kb

Figure 3.12

Identification of adjacent fragments to the 1.5 kb EcoRI fragment in the DNA ligase region of the T4 genome using a labelled probe made from λlig6-2 DNA. Tracks 1-4 are from an ethidium bromide stain, 1% agarose gel and 5-8, an autoradiograph of a nitrocellulose filter derived from this gel after hybridisation to the labelled probe. Track 1 contains R.HindIII digested T4 alc7 DNA; tracks 2 and 3 contain R.EcoRI digested T4 alc7 DNA, the former being a complete and the latter a partial digest; track 4 contains R.EcoRI digested T4 alc4 DNA. Estimates of fragment sizes are indicated.

Figure 3.12

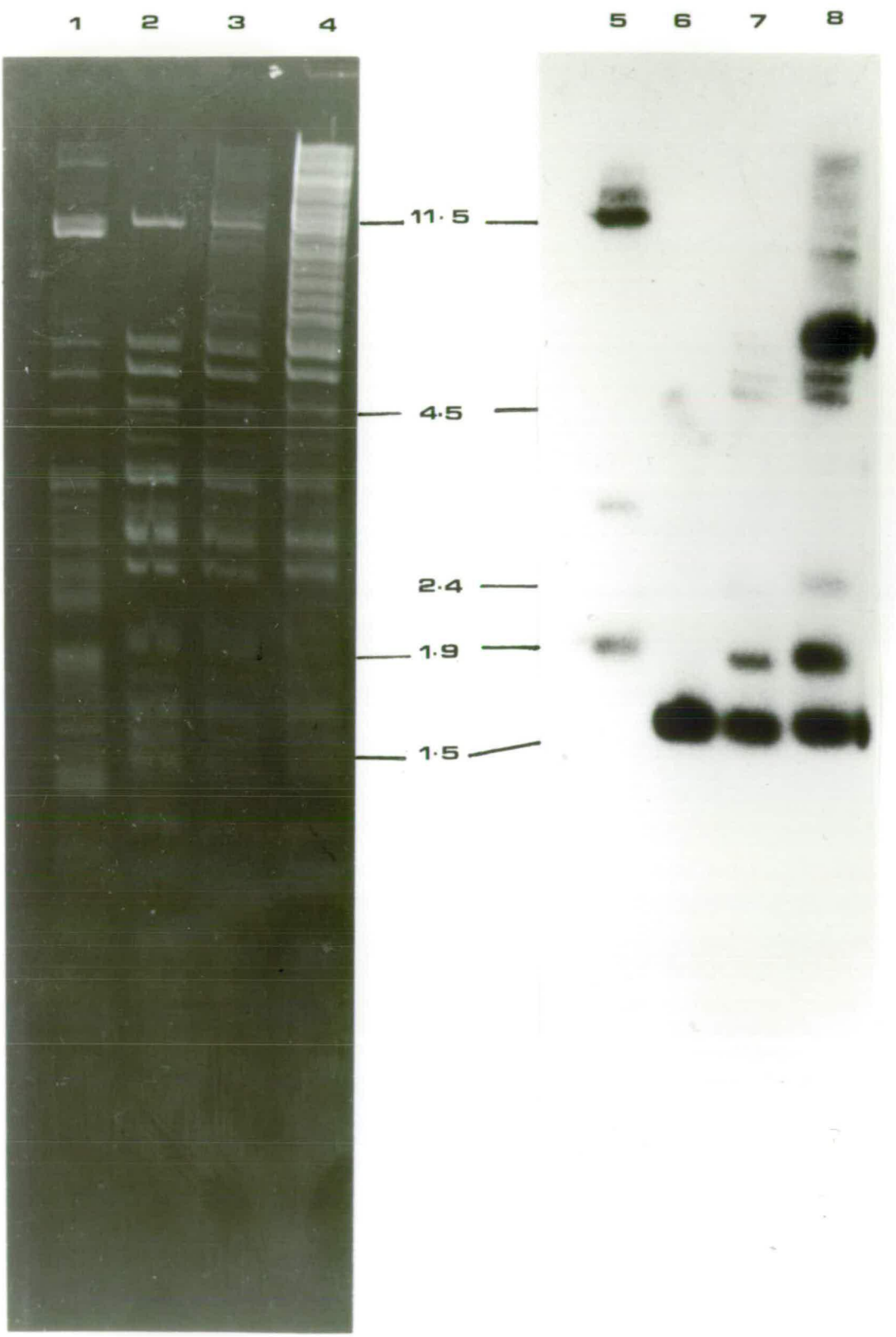
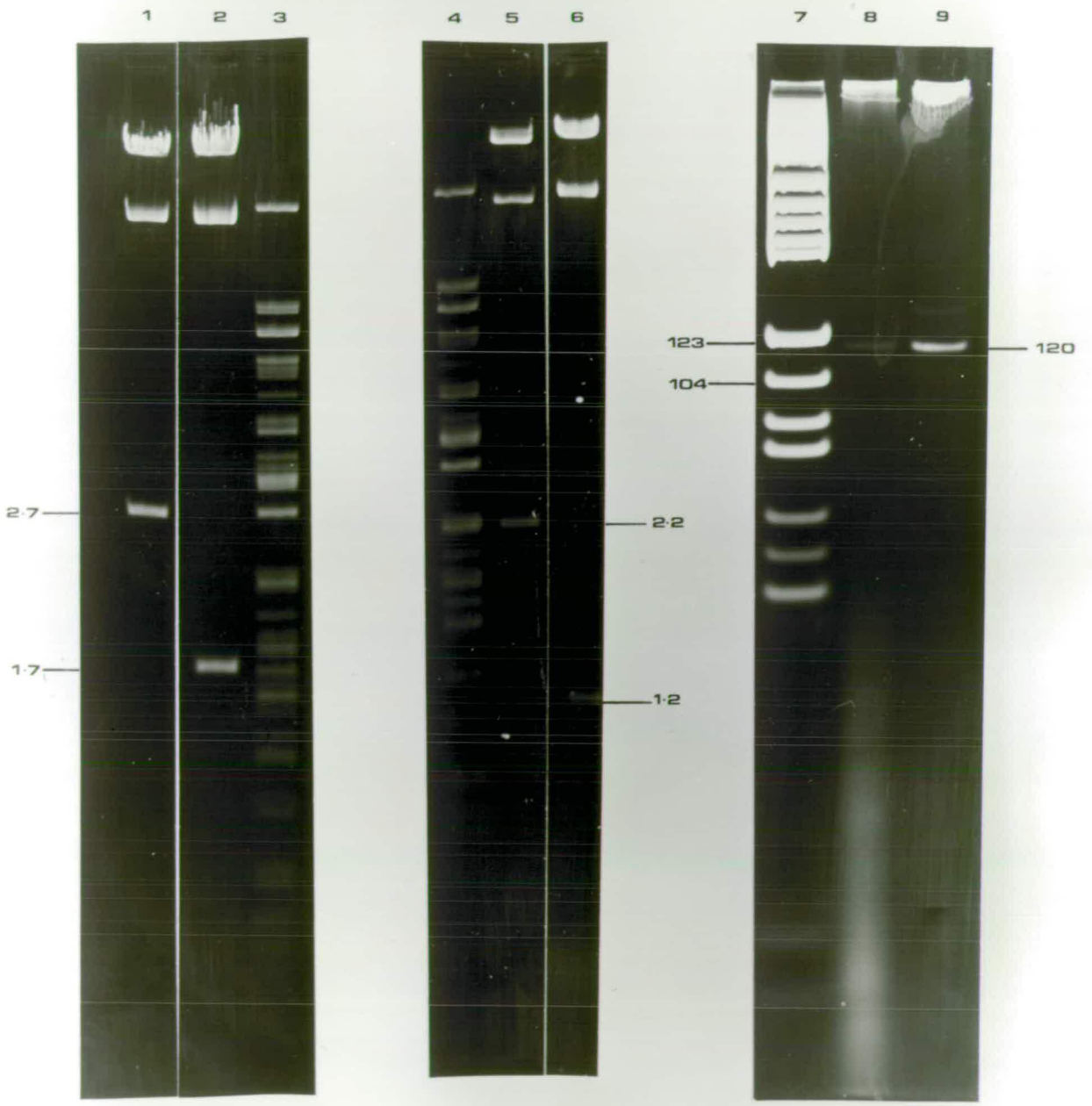


Figure 3.13

Structural analysis of λT4 recombinants carrying EcoRI fragments from the td-DNA ligase region. Tracks 1-6 are from ethidium bromide stained 1% agarose gels and 7-9 from an ethidium bromide stained 10% polyacrylamide gel. Track 1 contains DNA from 607/10, a recombinant carrying the 2.7 kb deletion derivative of the 3.1 kb EcoRI fragment; 2, DNA from 607/3, which carries the 1.7 kb EcoRI fragment; 3 and 4, T4 JW819 DNA; 5, DNA from 607/67, which carries the 2.2 kb EcoRI fragment; 6, DNA from 607/5, which carries the 1.1 kb EcoRI fragment; 7, pBR322 DNA; 8 and 9, DNA from 607/4 and 607/24, which carry the 0.12 kb fragment. The DNA in track 7 is digested with R.HaeIII and that in all other tracks by R.EcoRI. Estimates of fragment sizes in kb for tracks 1-6 or base pairs for 7-9 are indicated.

Figure 3·13



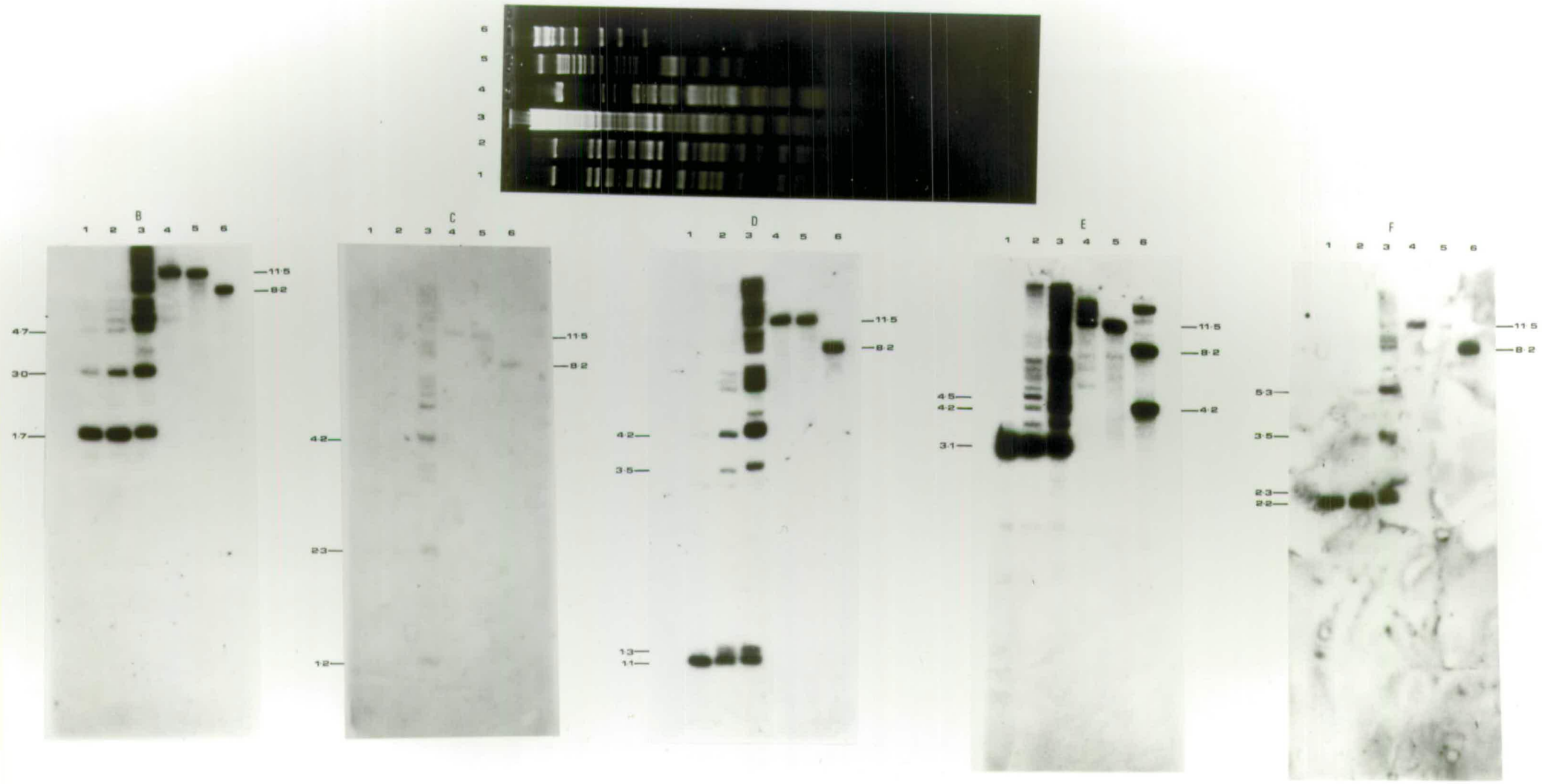
EcoRI fragment, behaved as if it included a T4 DNA fragment from a different region of the genome and was presumably derived from an impurity in the 9 kb HindIII/PstI fragment preparation. No recombinants carrying a 1.3 kb EcoRI fragment were recovered.

Although the 1.1, 1.7 and 2.2 kb EcoRI fragments were expected from the data obtained from the 9 kb HindIII/PstI fragment probe (see above), the 0.12 and 2.7 kb EcoRI fragments were not. The 0.12 kb fragment was too small to have been detected in the 9 kb HindIII/PstI fragment probe experiment, although λ derivatives carrying this fragment showed weak hybridisation to a labelled probe, made from T4 DNA, in a plaque hybridisation assay. This fragment has been visualised on a 10% polyacrylamide gel and was sized by comparison to a R.HaeIII digest of pBR322 DNA (see Figure 3.13). A labelled probe made from the DNA of a λ recombinant carrying the 2.7 kb EcoRI fragment gave positive hybridisation to a band of 3.1 kb in a complete R.EcoRI digest of T4 DNA transferred to a nitrocellulose filter and not to one of 2.7 kb (Figure 3.14). Restriction endonuclease analysis showed that this 2.7 kb EcoRI fragment carried the internal R.BglII site, but lacked the expected R.PstI site contained within the 3.1 kb EcoRI fragment (see Figure 3.15). Thus the 2.7 kb fragment behaves like a deletion derivative of the 3.1 kb EcoRI fragment, which retains both ends and the internal R.BglII site, but lacks the internal R.PstI site. This notion was further supported by the observation that T4 g 31 amber mutants could rescue wild-type markers from the 2.7 kb EcoRI fragment. The isolation of this deletion derivative was fortuitous since it was not expected to be wholly contained within the 9 kb HindIII/PstI fragment. Presumably it owes its origin to the presence of some 11.5 kb HindIII fragments in the 9 kb HindIII/PstI preparation.

Figure 3.14

Identification of adjacent EcoRI fragments in the td-DNA ligase region of the T4 genome using labelled probes. A: ethidium bromide stained 1% agarose gel; B-F: autoradiographs derived from this or similar gels, after hybridisation to the labelled probes. Tracks 1, 2 and 4-6 contain T4 JW819 DNA and track 3 T4 alc4 DNA. The DNA in tracks 1-3 is digested with R.EcoRI, that in track 2, only partially; by R.HindIII in track 4; by R.PstI in track 5; and by R.BglII in track 6. The labelled probes used were derived from the DNA of λT4 recombinants carrying the 1.7 kb EcoRI fragment in B; 0.12 kb EcoRI fragment in C; 1.1 kb EcoRI fragment in D; 2.7 kb deletion derivative of the 3.1 kb EcoRI fragment in D; and 2.2 kb EcoRI fragment in E. Estimates of fragment sizes in kb are indicated.

Figure 3·14


A
6
5
4
3
2
1
B
1 2 3 4 5 6
11·5
8·2
4·7
3·0
1·7
C
1 2 3 4 5 6
11·5
8·2
4·2
2·3
1·2
D
1 2 3 4 5 6
11·5
8·2
4·2
3·5
1·3
1·1
E
1 2 3 4 5 6
11·5
8·2
4·2
4·5
4·2
3·1
F
1 2 3 4 5 6
11·5
8·2
5·3
3·5
2·3
2·2

Th order of the EcoRI fragments identified within the td-DNA ligase region of the T4 genome was deduced from the hybridisation studies involving labelled probes made from the DNA of representatives of each of the five classes of λ recombinant derived from the region, and partial R.EcoRI digestsof T4 DNA (Figure 3.14). The sizes of partial products of R.EcoRI digested T4 DNA showing positive hybridisation to the probes, were determined and used to identify the sizes of adjacent EcoRI fragments. These data indicated the unambiguous order of EcoRI fragments within the td-DNA ligase region, shown in Figure 3.15.

A comparison of the EcoRI fragment order shown and the locations of restriction endonuclease targets in the region determined by other workers (Hangii and Zachau, 1980; Kiko et al, 1979), predicted that the 2.2 kb EcoRI fragment should contain an R.SmaI and an R.XbaI site and that the 1.7 kb EcoRI fragment should contain an R.KpnI site, if the order determined here was in agreement. The DNA from representatives of all classes of λ recombinants involved in the region, were digested with R.SmaI, R.KpnI and R.XbaI to test these predictions. R.SmaI and R.XbaI only cut the 2.2 kb EcoRI fragment and R.KpnI only cut the 1.7 kb EcoRI fragment as predicted, thus giving support to the order of EcoRI fragments within the td-DNA ligase region presented here.

d) Location of genes within the td-DNA ligase region

(a) g 63

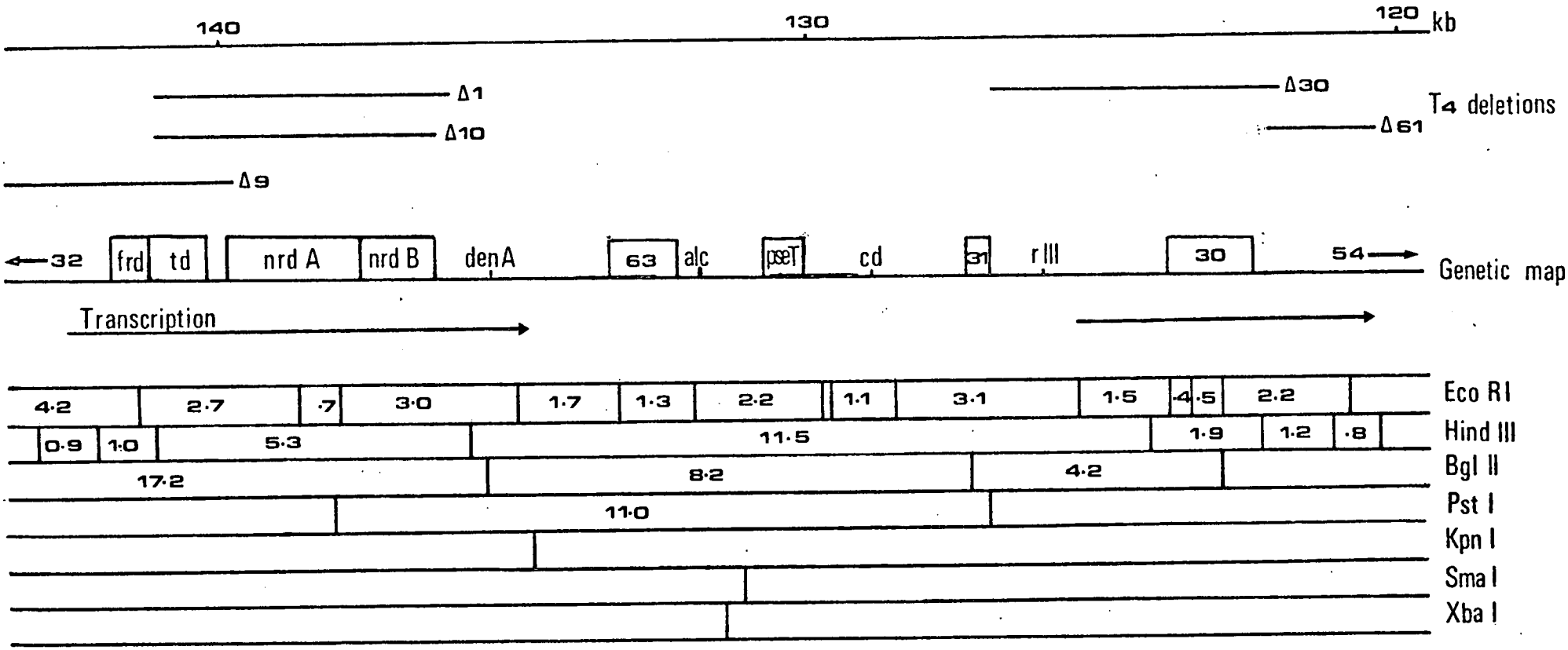
Representatives of each class of recombinant were screened by marker-rescue using two g 63 amber mutants, but with negative results. It seems likely that g 63 is carried on the 1.3 kb EcoRI fragment that was not recovered in the cloning of EcoRI fragments derived from

Figure 3.15

Organisation of the frd-g30 region of the T4 genome.

The figure shows:- The T4 genome: physical distance in kilobase pairs from the rIIA-rIIB join - 0 (Wood and Revel, 1976); T4 deletion mutants and their map positions determined by hetero-duplex mapping (Homyk and Weil, 1974; G.G. Wilson pers. comm.); a genetic map of T4 showing the gene order in the frd-g30 region: rectangles represent minimum lengths of genes whose products have been identified and sized by SDS polyacrylamide gel electrophoresis (see Wood and Revel, 1976); the direction of transcription as determined in this paper and Wilson and Murray (1979); a restriction map of the region, showing the position of targets for R.EcoRI, R.HindIII, R.BglII, R.PstI, R.KpnI, R.SmaI and R.XbaI. The unlabelled EcoRI fragment has a size of 120 base pairs. The 0.8 kb HindIII fragment has been identified in other experiments (A.J. Mileham, unpublished data).

Figure 3·15


140
130
120
kb
Δ1
Δ10
Δ9
Δ30
Δ61
T4 deletions
←32
frd
td
nrd A
nrd B
denA
63
alc
pseT
cd
31
rIII
30
54→
Genetic map
Transcription
4·2
2·7
·7
3·0
1·7
1·3
2·2
1·1
3·1
1·5
·4
·5
2·2
Eco RI
0·9
1·0
5·3
11·5
1·9
1·2
·8
Hind III
17·2
8·2
4·2
Bgl II
11·0
Pst I
Kpn I
Sma I
Xba I

the 9 kb HindIII/PstI fragment. This notion is supported by the fact that alignment of the genetic and physical maps of the td-DNA ligase region predicts that the 1.3 kb EcoRI fragment should contain at least part of g 63 (see Figure 3.15).

(b) pseT

Probes made from the DNA of representatives of each class of λ recombinant involved in the region were used to determine the position of the pseT gene within the physical map. They were used to detect the presence of homologous sequences in non-glucosylated T4 DNA prepared from strains carrying either a point mutation in the pseT gene, or a deletion that removes all known markers within the pseT gene, but which does not involve the alc gene (Sirotkin et al, 1978). It was found that the 2.2, 1.1 and 0.12 kb EcoRI fragments were missing in the deletion strain but present in the point mutant strain (Figure 3.16). No hybridisation was seen to fragments smaller than 2.2 or 1.1 kb, when the 2.2 and 1.1 kb fragments were used as probes, and very little to larger fragments, implying that the deletion removes most of each fragment. Thus the pseT gene must lie within the limits of these three fragments. Again this position is predicted from alignment of physical and genetic maps of the region (see Figure 3.15). The alc gene is not affected by the pseT deletion and so probably lies outside the 2.2 kb EcoRI fragment. This would mean that the alc gene is at least mainly within the 1.3 kb EcoRI fragment.

(c) g 31

Representatives of each class of λ recombinants involved in the region were screened for marker rescue with two g 31 amber mutants. While all other classes gave negative results, recombinants carrying

Figure 3.16

Identification of the EcoRI fragments in the pseT region of the T4 genome. A: ethidium bromide stained 1% agarose gel; B-F: autoradiographs derived from this or similar gels, after hybridisation to the labelled probes. Track 1 contains T4 pseT-1 DNA; 2, T4 pseTΔ1 DNA; 3 and 5, T4 JW819 DNA; 4, T4 alc4 DNA. The DNA in tracks 1-4 are digested with R.EcoRI and that in track 5 with R.HindIII. The labelled probes used were derived from the DNA of λT4 recombinants carrying the 1.7 kb EcoRI fragment in B; 0.12 kb EcoRI fragment in C; 1.1 kb EcoRI fragment in D; 2.7 kb deletion derivative of 3.1 kb EcoRI fragment in E; and 2.2 kb EcoRI fragment in F. Estimates of fragment sizes in kb are indicated.

Figure 3·16

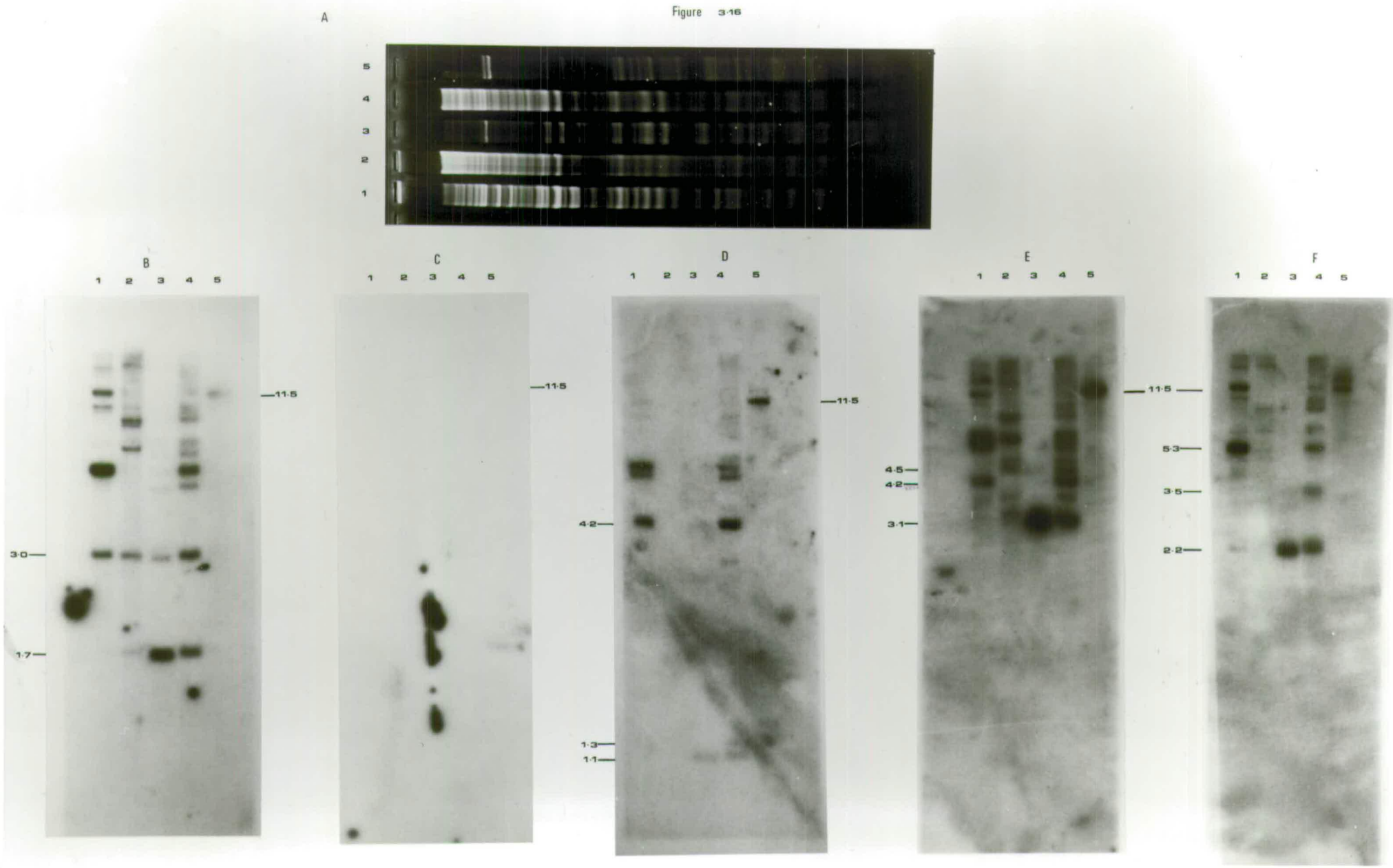

A
5
4
3
2
1
B
1 2 3 4 5
11·5
3·0
1·7
C
1 2 3 4 5
11·5
D
1 2 3 4 5
11·5
4·2
1·3
1·1
E
1 2 3 4 5
11·5
4·5
4·2
3·1
F
1 2 3 4 5
11·5
5·3
3·5
2·2

Table 3.3 Marker rescue of g 31 by the 2.7 kb deletion derivative of the 3.1 kb EcoRI fragment

T4 g 31 amber	Phage	Titre of phage on hosts W3101/λ	CR63	Efficiency of plating on non-permissive host
N54	607/10	1.2×10^4	1.5×10^9	8.0×10^{-6}
	607/17	9.0×10^3	5.0×10^8	1.8×10^{-5}
S54	607/10	3.0×10^3	7.0×10^7	4.3×10^{-5}
	607/17	2.0×10^3	1.0×10^8	2.0×10^{-5}
			average	2.2×10^{-5}

607/10 and 607/17 were independent isolates of derivatives from the ligation mix of R.EcoRI restricted λNM607 and isolated T4 9 kb HindIII/PstI fragment which carry the 2.7 kb deletion derivative of the 3.1 kb EcoRI fragment. W3101/λ is suppressor-free and λ resistant and so is non-permissive to both λ and T4 amber mutants. CR63 is λ resistant, but permissive for T4 amber mutants. Marker rescue was carried out as in Section 2.2 (h)(iii).

the 2.7 kb deletion derivative of the 3.1 kb EcoRI fragment, rescued both markers with an efficiency of about 10^{-4}-10^{-5} (see Table 3.3). This indicates that the 2.7 kb deletion derivative of the 3.1 kb EcoRI fragment carries at least part of g 31 and confirms the position of the 3.1 kb EcoRI fragment within the td-DNA ligase region of the T4 genome (see Figure 3.15).

(d) rIII

The rIII gene maps slightly anticlockwise of g 31 on the T4 map (Revel and Lielaussis, 1978; see Figure 1.2) and is expected to be contained within the 3.1 kb EcoRI fragment from the alignment of physical and genetic maps of the td-DNA ligase region (see Figure 3.15). Selzer et al (1978) have found that the related T4 rII genes cannot be cloned intact on plasmid vectors and that host cells containing a recombinant plasmid carrying part of the rIIA gene, which coded for a truncated rIIA polypeptide, grew very poorly. Suggestive evidence that at least part of the rIII gene is present on the 2.7 kb deletion derivative of the 3.1 kb EcoRI fragment comes from the observation that cells harbouring a pBR325 derivative carrying this 2.7 kb EcoRI fragment, grow much more slowly than cells containing a pBR325 derivative carrying the 1.7 kb EcoRI fragment. The plasmid derivatives carrying the 2.7 kb Eco fragment also die quickly so that cells carrying them cannot be isolated from a culture after a few days growth.

4. DISCUSSION

Experiments described in this thesis provide genetic, functional and physical evidence that the T4 genes td and frd have been cloned in λ vectors. Direct enzyme assays, to detect the products of these genes in cells infected by appropriate recombinant phages, have not been made, although L. Meade (pers. comm.) has shown that λtd-1 does indeed induce a thymidylate synthetase activity on infection of E.coli cells. The cloning of these two genes has allowed the alignment of the physical and genetic maps of the td region of the T4 genome. Functional analyses of λtd recombinants have shown that the direction of transcription of the structural genes in the td region is anticlockwise with respect to the T4 genetic map, as expected for early T4 genes (see Wood and Revel, 1976). The direction of transcription of the neighbouring DNA ligase gene has also been shown to be anticlockwise (Wilson and Murray, 1979), thus it seems likely that the other genes in the frd-DNA ligase region will be transcribed in the same direction, as they are all early or quasi-late genes.

The whole frd-DNA ligase region of the T4 genome comprises four HindIII and fourteen EcoRI fragments. The sizes of the EcoRI fragments contained within the 11.5 kb HindIII fragment and the sections of the two overlapping EcoRI fragments that are carried by the 11.5 kb HindIII fragment, add up to 11.5 kb. This indicates that any remaining EcoRI fragments in this region must be very small, certainly smaller than the 120 bp fragment which was detected when partial R.EcoRI digests of T4 DNA were hybridised against various labelled probes derived from the region. The entire td-DNA

ligase region of the T4 genome has been estimated to be 18 kb long by heteroduplex analysis (see Introduction). The size of the same region can be estimated from the physical data in this thesis. The region from the R.EcoRI site within the 1.0 kb HindIII fragment just to the left of the td gene, to the R.HindIII site within the 2.2 kb EcoRI fragment just to the right of the DNA ligase region, comprises 19.0 kb (see Figure 3.15). Thus these two physical estimates for the size of this region of the T4 genome are in good agreement indicating that any additional restriction fragments in the region must be very small. The hybridisation of T4 DNA, digested partially with R.EcoRI, against labelled probes derived from the frd-DNA ligase region, has led to the establishment of an unambiguous order of EcoRI fragments within this region.

The molecular cloning of virulent phages has intrinsic problems. Such phages are programmed to turn off host gene expression, degrade host DNA, take over the cells metabolic machinery and eventually lyse the cell. Thus it is unlikely that expression of such functions from DNA derived from such phages carried on plasmid vectors could be tolerated by the host cell. The use of λ vectors may overcome some but not all of these problems, for example cell lysis functions may be amenable to molecular cloning on a λ vector. In keeping with the notion that it may not be possible to clone DNA fragments carrying such deleterious functions, recombinants carrying the 11.5 kb HindIII, the 1.3 kb EcoRI or the intact 3.1 kb EcoRI fragment contained within the frd-DNA ligase region of the T4 genome, have never been isolated. Several deleterious functions may be encoded by this region of the T4 genome: one carried by the 1.3 kb EcoRI fragment, another involved in the

region deleted from the 3.1 kb EcoRI fragment in its 2.7 kb derivative and since the EcoRI fragments of the region have only been cloned singly, still more deleterious functions may need two or more contiguous EcoRI fragments for their expression. Deleterious functions carried by the EcoRI fragments mentioned would automatically explain the failure to clone the 11.5 kb HindIII fragment.

Marker rescue tests involing g 63 amber mutants and all the cloned EcoRI fragments in the frd-DNA ligase region failed to give positive results, suggesting that at least the part of g 63 covered by the mutations used, is not present on the fragments cloned. Alignment of the physical and genetic maps of the region (see Figure 3.15), predicts that at least part of g 63 lies within the 1.3 kb EcoRI fragment that has so far proved impossible to clone. It seems likely that g 63 is at least partly carried by the 1.3 kb EcoRI fragment. If g 63 is carried on this fragment, it does not necessarily mean that gp 63 is deleterious for E.coli or λ functions. The use of T4 DNA carrying a g 63 amber mutation to produce λT4 recombinants could clarify the issue in a similar way to the demonstration that the expression of gp 32 prevents the cloning of the 4.2 kb EcoRI fragment that carries g 32 (H. Kirsch pers. comm.) If it proves possible to recover the 1.3 kb EcoRI fragment in recombinants derived from T4 g 63 am DNA, then it would seem that gp 63 has a deleterious effect on E.coli or λ functions, perhaps by the production of abberant RNA molecules by its RNA ligase activity. However if recombinants carrying the 1.3 kb EcoRI fragment are not recovered from T4 DNA of this type, then it would seem likely that an unknown deleterious function is carried on this fragment in addition to g 63. This function could be specified by

the alc gene whose product binds to the E.coli RNA polymerase to provide the HMC template specificity of late T4 transcription (Snyder et al, 1976). The alc gene is predicted to lie within the 1.3 kb EcoRI fragment since the 2.2 kb EcoRI fragment is almost entirely missing from the DNA of the T4 pseT deletion strain whose alc gene is wild type (Sirotkin et al, 1977) and the alc gene maps clockwise of the pseT gene on the T4 genetic map (see Figure 1.2). Although the T4 DNA used to generate recombinants which were expected to include those carrying the 1.3 kb EcoRI fragment, carried an alc$^-$ allele, it is possible that any altered polypeptide produced could still bind to the host RNA polymerase and exert a deleterious effect.

The 2.7 kb deletion derivative of the 3.1 kb EcoRI fragment rescues two markers in g 31, indicating that at least part of g 31 is carried on this fragment. The alignment of the gnetic and physical maps of the region predicts that both g 31 and the rIII gene should be carried by the 3.1 kb EcoRI fragment (see Figure 3.15). Suggestive evidence for the presence of at least part of the rIII gene on the 2.7 kb deletion derivative, comes from the fact that the presence of this fragment on a plasmid vector in a host cell causes the host strain to grow poorly. The same phenomenon has been observed for plasmids carrying part of the related rIIA gene (Selzer et al, 1978). Marker rescue tests using rIII mutants and the plasmid derivative carrying the 2.7 kb deletion derivative of the 3.1 kb EcoRI fragment, could detect the presence of the whole or part of the rIII gene on this fragment. This test would be complicated by the fact that rIII mutations are not conditionally lethal. The transfer of the 2.7 kb deletion derivative of the 3.1 kb

EcoRI fragment into a λ vector whose cloning site is in a position which ensures the expression of functions carried on inserted DNA fragments, could demonstrate whether or not g 31 and the rIII gene are carried on this fragment. The products of genes 31 and rIII could be identified using such phages, assuming that their structural genes are intact on the 2.7 kb deletion derivative of the 3.1 kb EcoRI fragment. These products could then be identified as bands present amongst labelled polypeptides derived from the infection of UV irradiated cells by such recombinant phage or wild type T4, after polyacrylamide gel electrophoresis, but absent amongst those derived from infections involving the λ vector or T4 strains carrying amber mutations in g 31 or the rIII gene. The apparent Mr of gp 31 has been estimated as 16,000 (Castillo et al, 1977).

In addition to identifying gps frd, td and nrdA amongst the polypeptides encoded by λtd recombinants, gene expression studies have also revealed the presence of two unknown T4 gene products specified by the same recombinants. Polypeptide X is only specified by λtd recombinants carrying the 3.0 kb EcoRI fragment, but it is not known whether or not its coding sequence is entirely within this fragment. The analysis of polypeptides derived from UV irradiated cells infected by λT4 recombinants carrying only this 3.0 kb EcoRI fragment would clarify this matter. Such a recombinant phage has been isolated but not used as described above. Alignment of genetic and physical maps of the region predicts that the denA lies at the g 63 end of the 3.0 kb EcoRI fragment (see Figure 3.15), and so denA could possibly be the structural gene for polypeptide X. However polypeptide X is specified by the relevant λtd recombinants derived from either $denA^+$ or $denA^-$ DNA. The nature of the denA

mutation used is not known but it is possible that a mutation that inactivates the function of an enzyme has no effect on its apparent Mr. The size of gp denA is also unknown. A recombinant carrying both 3.0 and 1.7 kb EcoRI fragments should certainly carry the denA gene and could clarify whether or not polypeptide X is the denA product. If the denA mutation affects the size of gp denA, then a comparison of labelled polypeptides derived from UV irradiated cells infected with recombinant phage carrying the 3.0 and 1.7 kb EcoRI fragments derived from both denA$^+$ and denA$^-$ T4 DNA, should show a difference in one polypeptide band after electrophoresis. If polypeptide X is the only T4 polypeptide induced by such recombinant phage, and no difference in the band pattern is seen between the polypeptides induced by phages derived from denA$^+$ and denA$^-$ T4 DNA, then it would be likely that the denA mutation is missense and polypeptide X, the product of the denA gene. However if a difference in band pattern is seen, then that band would represent gp denA and polypeptide X would be the product of a previously unidentified T4 gene.

Polypeptide Y is encoded by the 2.4 kb segment of DNA common to the 2.7 kb EcoRI and 5.3 kb HindIII fragments, which places the coding sequence for this polypeptide between the td and nrdA genes. As no T4 gene is known in this position, the structural gene for this polypeptide must be a previously unknown and presumably non-essential T4 gene, since no amber mutations are known to map in this region and this region can be deleted without affecting phage viability (Homyk and Weil, 1974).

The nature of polypeptides X and Y is unknown, but since their coding sequences lie in a cluster of genes whose products are

involved in nucleotide metabolism, it seems reasonable to assume that polypeptides X and Y are also involved in such processes.

The clear plaque morphology of λtd recombinants carrying either the 3.0 kb EcoRI or 5.3 kb HindIII fragments must be due to a function encoded by the 2.2 kb DNA segment, common to both fragments, which interferes with the establishment or maintenance of lysogeny. This function is unlikely to involve the nuclease activity of the denA product, as the clear plaque phenotype occurs in recombinants generated from both denA$^+$ and denA$^-$ T4 DNA (denA mutants are defined as nuclease deficient), and cannot involve polypeptide X as it is not specified by the 5.3 kb HindIII fragment. The only known T4 genes whose products are likely to be carried by the 3.0 kb EcoRI and 5.3 kb HindIII fragments are nrdA and nrdB, and there is no obvious reason why the products of these genes should interfere with λ lysogeny. Turbid derivatives of these clear plaque recombinants can be isolated and these do not involve any gross changes in DNA structure since both the original phage and their turbid derivatives show identical restriction patterns. Derivatives which carry an amber mutation in the gene specifying the function which interferes with lysogeny could allow the identification of this function by altering the mobility of a polypeptide band after polyacrylamide gel electrophoresis of labelled polypeptides derived from UV irradiated cells infected by λtd recombinants of clear plaque morphology and derivatives carrying such amber mutations. Such amber mutants would have a clear plaque morphology on a suppressor strain and a turbid plaque phenotype on a suppressor-free strain. Amber mutants of this type have been repeatedly looked for without success.

Evidence presented in this thesis suggests that at least two T4 promotors have been cloned from the frd-DNA ligase region. A T4 promotor is present on the 2.7 kb EcoRI fragment and can be used to initiate the transcription of the td gene as thyA E.coli cells, lysogenic for λT4 recombinants carrying this 2.7 kb EcoRI fragment, in either orientation, are thymine independent. As this effect is seen for λtd phages of both possible orientations, it is unlikely that the transcription of the td gene, in the lysogenic state, is initiated from weak constitutive promotors on the λ chromosome. The nrdB product was not detected in the gene expression studies described here, but B-M. Sjoberg (pers. comm.) has detected its presence on a Q^-, S^- derivative of λtd-611. The T4 genes carried on λtd-611 are transcribed in the same direction as that initiated from the P'_R promotor and thus, while the Q^-, S^- derivative of this phage could possible weakly transcribe its T4 genes from the P_R promotor, it is more likely that the transcription of the T4 genes here is from an included promotor. This promotor may or may not be the same as that that serves the td gene. A derivative of the λtd-611 Q^-, S^- phage that lacks the 2.7 kb EcoRI fragment could resolve the issue; if such a phage still specifies gp nrdB activity then a promotor for the nrdB gene must exist downstream from the td gene.

There is some debate over the presence of a promotor on the 1.0 kb HindIII fragment that carries the frd gene. Hangii and Zachau (1980) claimed that the R.HindIII target that forms the left-hand end of the 1.0 kb HindIII fragment (see Figure 3.15), lies within the frd gene. The authors concluded this because T4 DNA cut with R.HindIII failed to specify gp frd in a coupled

transcription/translation system. Genetic and biochemical evidence presented in this thesis demonstrates that the 1.0 kb HindIII fragment carries an intact and functional frd gene. In the light of this evidence an alternative explanation is that the HindIII site to the left of the frd gene must separate the frd gene from its promotor. However the frd gene can be expressed in cells lysogenic for λ derivatives carrying the 1.0 kb HindIII fragment in either of the two possible orientations, and enables such lysogens to grow in concentrations of trimethoprim that inhibit the growth of non-lysogenic cells.

It is unlikely that the transcription of the frd gene in lysogens can be from non-T4 promotors. The weak constitutive rightwards λ promotor detected by Hopkins et al (1976), could explain frd expression in lysogens for a λfrd$^+$ recombinant carrying the 1.0 kb HindIII fragment in the r orientation, and while Ptashne (1978) has evidence for a weak constitutive λ leftwards promotor, D. Burt (pers. comm.) has not detected a promotor between the shnλ3 site and att, thus the expression of the frd gene in lysogens carrying λfrd$^+$ phage of l orientation, cannot be from this leftwards promotor, since the shnλ3 site is used as the cloning site in λNM540. It is also possible that R.HindIII does indeed cleave the frd promotor, and that insertion of the 1.0 kb HindIII fragment into λNM540 produces a hybrid promotor site, at least at the right-hand site of the insertion if lysogenic expression of l orientation λfrd$^+$ phages from non-T4 promotors is to be explained, and this can then initiate the transcription of the frd gene.

Thus at least the frd and td genes have contiguous promotors,

indicating that the genes in the td region of the T4 genome may not necessarily be coordinately transcribed as one might intuitively expect.

The alignment of physical and genetic maps of the region and the hybridisation data involving the pseT mutant DNAs, predicts that the pseT gene is confined to a region of the T4 genome specified by the 2.2, 0.12 and 1.1 kb EcoRI fragments. It is not known whether one, two or all three fragments are involved. This problem could be resolved in two basic ways: the transfer of each of these fragments from the immunity region of the λNM607 derivatives, into a λ vector, the position of whose cloning site assures the expression of functions carried on the cloned DNA fragment, and the isolation of new recombinants carrying two or three of these fragments contiguously. The isolation of such phage would be greatly aided by the transfer of these EcoRI fragments into pBR325, as such derivatives could be used as probes in plaque hybridisation assays to pick up desirable λT4 recombinants. Once isolated such λT4 derivatives could be used in gene expression studies to identify any T4 polypeptides that they might encode, and extracts of cells infected by such recombinant phage could be assayed for polynucleotide kinase activity. Many attempts have been made to construct such derivitive phage and plasmid recombinants, but with little success; only the 0.12 kb fragment from this region has been successfully transferred to pBR325. This plasmid derivative has been used as a probe to detect recombinants carrying fragments from this region of the T4 genome, in plaque hybridisation tests, but no recombinants carrying either the 1.1 or 2.2 kb EcoRI fragments have been isolated.

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APPENDIX

Molecular Cloning of the T4 tk gene

Introduction

On infection T4 specifies the production of several enzymes involved in pyrimidine metabolism (see Section 1d), including a thymidine kinase. Hiraga et al (1967), first demonstrated this activity in T4 infected, thymidine kinase deficient (tdk) E.coli cells, and showed that the T4 induced enzyme differed from that of E.coli in heat stability. Chace and Hall (1973), reported the isolation of T4 mutants unable to induce thymidine kinase (tk), by a selective method. DNA containing the thymidine analogue 5-bromodeoxyuridine (BUdR) is light sensitive (Lion, 1970), and since BUdR is phosphorylated by the E.coli thymidine kinase (Okazaki and Kornberg, 1964), a step necessary for the incorporation of BUdR into DNA, cells or phage that specify an active thymidine kinase should be unable to grow in the presence of light. Thus Chace and Hall (1973), infected tdk^- E.coli cells with T4 in the presence of BUdR and light. They increased the sensitivity of selection by adding 5-fluorodeoxyuridine (FUdR), uridine and deoxyadenosine to the medium. FUdR inhibits both the E.coli and T4 thymidylate synthetase (Flacks and Cohen, 1959; Mathews and Cohen, 1963), so lowering the pool size of thymidylate precursors in the infected cell and therefore increasing the incorporation of BUdR into nascent DNA; uridine allows cell growth by ensuring that FUdR is not incorporated into nascent RNA molecules (Cohen et al, 1958), and deoxyadenosine was expected to inhibit the conversion of BUdR and FUdR to 5-bromouracil and 5-fluorouracil respectively in a similar way to which it inhibits the conversion of thymidine to thymine (Yagil and

Rosner, 1970); see Figure A.1 for a summary of these events). The authors found that all phages recovered from plaques that grew under the selective conditions were deficient in the induction of the T4 thymidine kinase and were thus tk⁻ mutants. All tk⁻ mutants isolated by Chace and Hall (1973) were allelic and mapped next to the rI gene (see Figure 1.2); indeed some tk⁻ mutants also had a rapid lysis phenotype and appeared to be deletions involving the tk and rI genes. Thus the tk gene is unlinked to other T4 genes whose products play a role in pyrimidine metabolism (see Hall et al, 1967). Thymidine kinase in induced at early times in T4 infections, and so the tk gene must be under early or quasi-late control (Chace and Hall, 1973; see Section 1e(i)). Chace and Hall (1975) demonstrated that the T4 tk locus was the structural gene for the T4 induced thymidine kinase, by showing that the enzyme induced by a T4 strain carrying an amber mutation in the tk gene, on infection of a tdk⁻ E.coli strain carrying an amber suppressor, was much more heat sensitive than that induced by wild type T4.

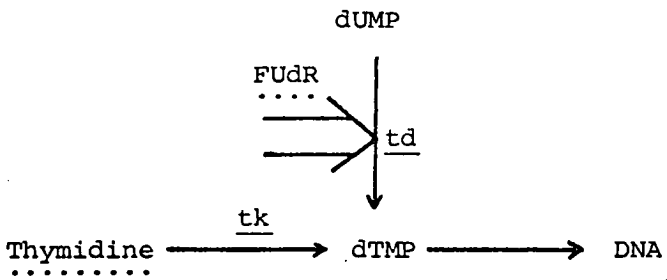
The role of thymidine kinase in T4 infection is not obvious since T4 tk⁻ mutants grow as well as T4 wild type phages on tdk⁻ E.coli strains (Chace and Hall, 1973), since thymidylate is still available from the breakdown of host DNA and the action of thymidylate synthetase. Presumably there must be environmental conditions or host strains in which the induction of the T4 thymidine kinase is beneficial for the formation of T4 DNA.

Igarashi et al (1967) showed that the E.coli tdk gene mapped between the galU and supF genes and was thus close to the attachment site of coliphage ø80. These authors found that they could pick up defective ø80 transducing phages carrying the tdk gene. These

Figure 4.1

The inhibitory effects of FUT medium on pyrimidine metabolism in T4 infected cells. The T4 genes whose products are involved in these processes are indicated beside the arrows. Compounds underlined with dotted line are ingredients of FUT medium.

Figure 4.1



phages could grow on tdk$^-$ hosts in medium containing FUdR, uridine and thymidine (FUT medium), as, although FUdR blocked the action of thymidylate synthetase, the thymidine kinase gene present on the transducing phages allowed the production of thymidylate from thymidine, and thus phage growth. It was hoped that λ recombinants carrying the T4 tk gene could be detected similarly.

2. Additional Materials and Methods

1. FUT Medium

L or BBL bottom agar supplemented with 25 µg/ml FUdR, 25 µg/ml uridine and 50 µg/ml thymidine.

2. Chemicals

FUdR was purchased from Sigma Chemical Company.

3. Bacterial strain

The tdk$^-$ strain used in this work was:

KY895 tdk-1, ilv-276 (Hiraga et al, 1967).

4. λT4 Recombinants

λT4 recombinants used here were generated from R.HindIII digested λNM540 and T4 alc1 DNA.

5. Identification of λ tk recombinants

0.2 ml of KY895 plating cells were mixed with 3 ml of BBL top layer agar, and poured onto a BBL based FUT medium plate. 0.01 ml drops of serial dilutions of λT4 recombinants were then applied to the top layer and the plates incubated overnight at 37^{o}C. λtk phages were scored next day as plaque forming units on a very weak bacterial lawn.

6. Formation of lysogens

Derivatives of KY895 lysogenic for λtk phages were constructed as described in Section 2.2d, in the absence of FUT medium.

3. Results

1. Isolation and genetic characterisation of λtk recombinants

Two independent phages, λtd-17 and -21, were isolated as presumptive λtd recombinants, as they formed plaques on the thyA E.coli strain in the absence of thymine. Examination of the DNA of these phages showed that they did not carry the td gene, as described below. Consideration of the biochemistry of pyrimidine metabolism in T4 infected cells, suggested that these recombinants could carry the T4 tk gene. The increased levels of thymidine kinase in cells infected with a λtk recombinant might provide an adequate amount of thymidylate for DNA production, from any thymidine present in such cells. Thus λtd-17 and -21 were tested for the ability to form plaques on the tdk^- host in the presence of FUT medium. Both recombinants formed plaques under these conditions whereas the λ vector and λtd recombinants failed to do so. Since only phages including a thymidine kinase gene can plate on tdk^- cells on FUT medium, λtd-17 and -21 were re-named λtk-1 and -2.

Derivatives of the tdk^- host lysogenic for the λtk phages were made in the absence of FUdR. These lysogens grew on L agar based FUT medium plates, whereas tdk^- derivatives lysogenic for the λ vector or λtd phages could not. The expression of cloned functions in the lysogenic state is suggestive evidence that the promotor for the structural gene for that function has also been cloned.

2. Physical characterisation of λtk recombinants

The DNA of both λtk recombinants was examined by agarose gel electrophoresis after digestion with R.EcoRI and R.HindIII. The DNA of both phages was identical with respect to the fragments of T4 DNA carried and the orientation of the insert. Both carried

Figure 4.2

Structural analysis of λtk recombinants. Ethidium bromide stained 1% agarose gel containing λtk2 DNA digested with R.HindIII in track 2, R.HindIII and R.EcoRI in track 3, and R.EcoRI in track 4. Track 1 contains R.HindIII digested λcI857 DNA. Estimates of fragment sizes in kb are indicated.

Figure 4·2

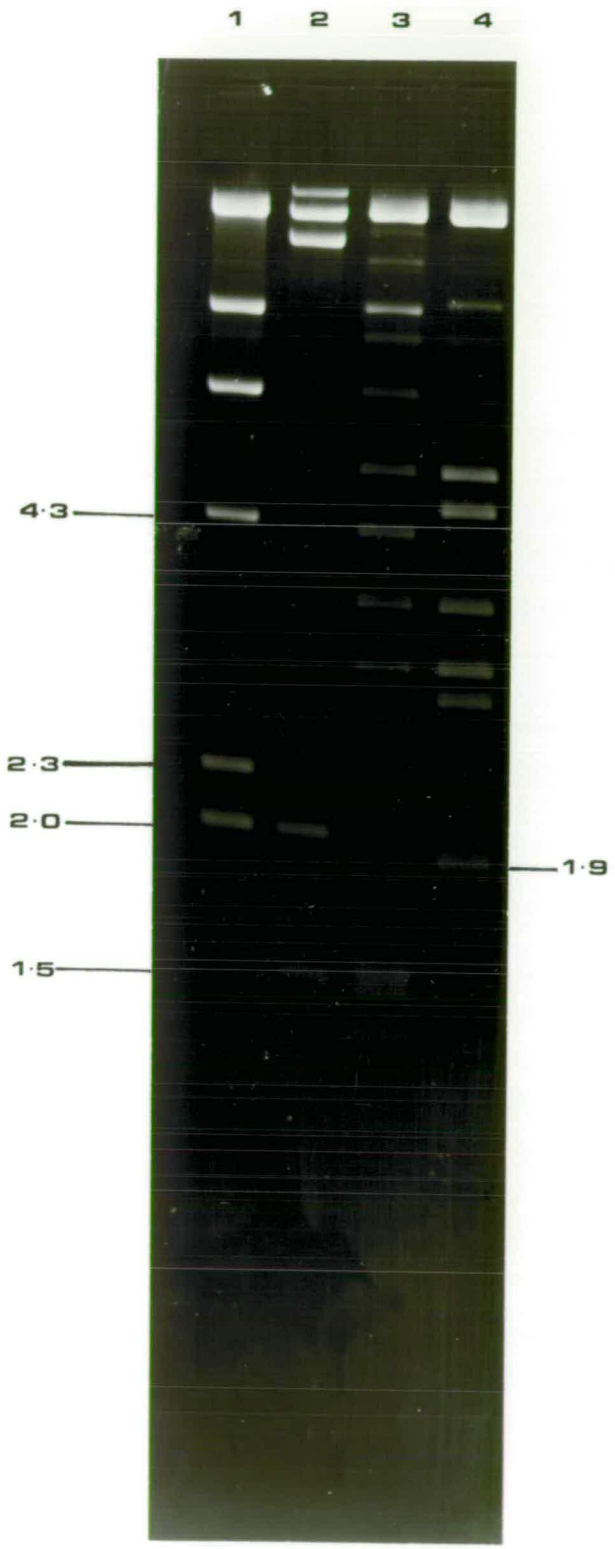


Figure 4.3

Restriction map of λtk-2 DNA is shown at the top. ▼ represent R.EcoRI targets, and Δ, R.HindIII targets. The sizes of fragments are in kb. The map at the bottom shows the structural organisation of the tk region of the T4 genome. The sizes of fragments are in kb.

Figure 4.3

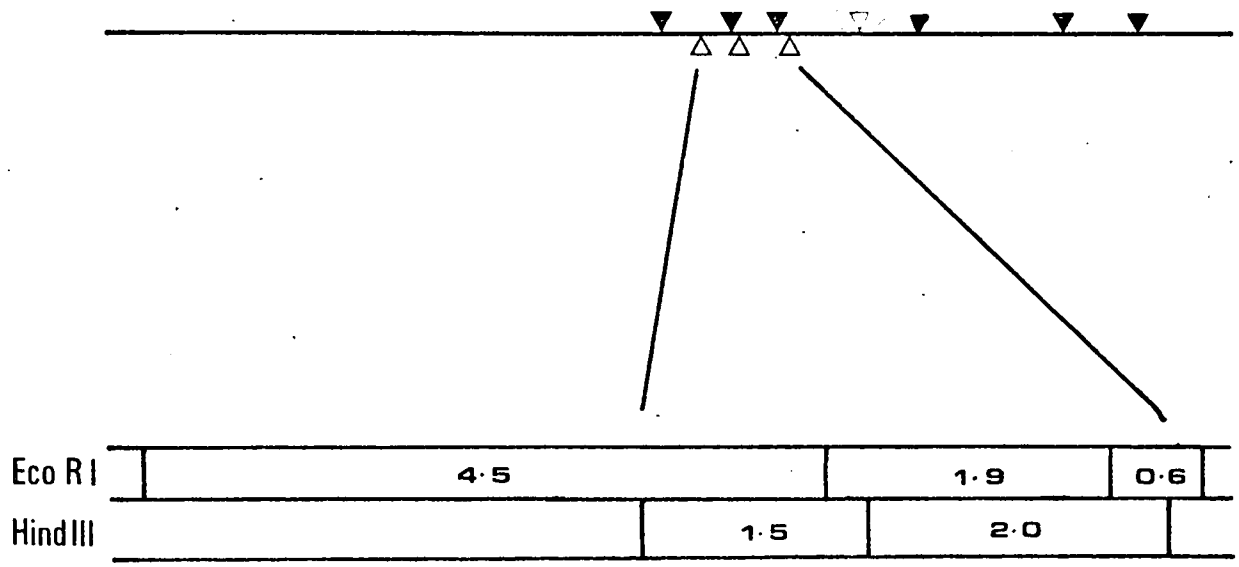
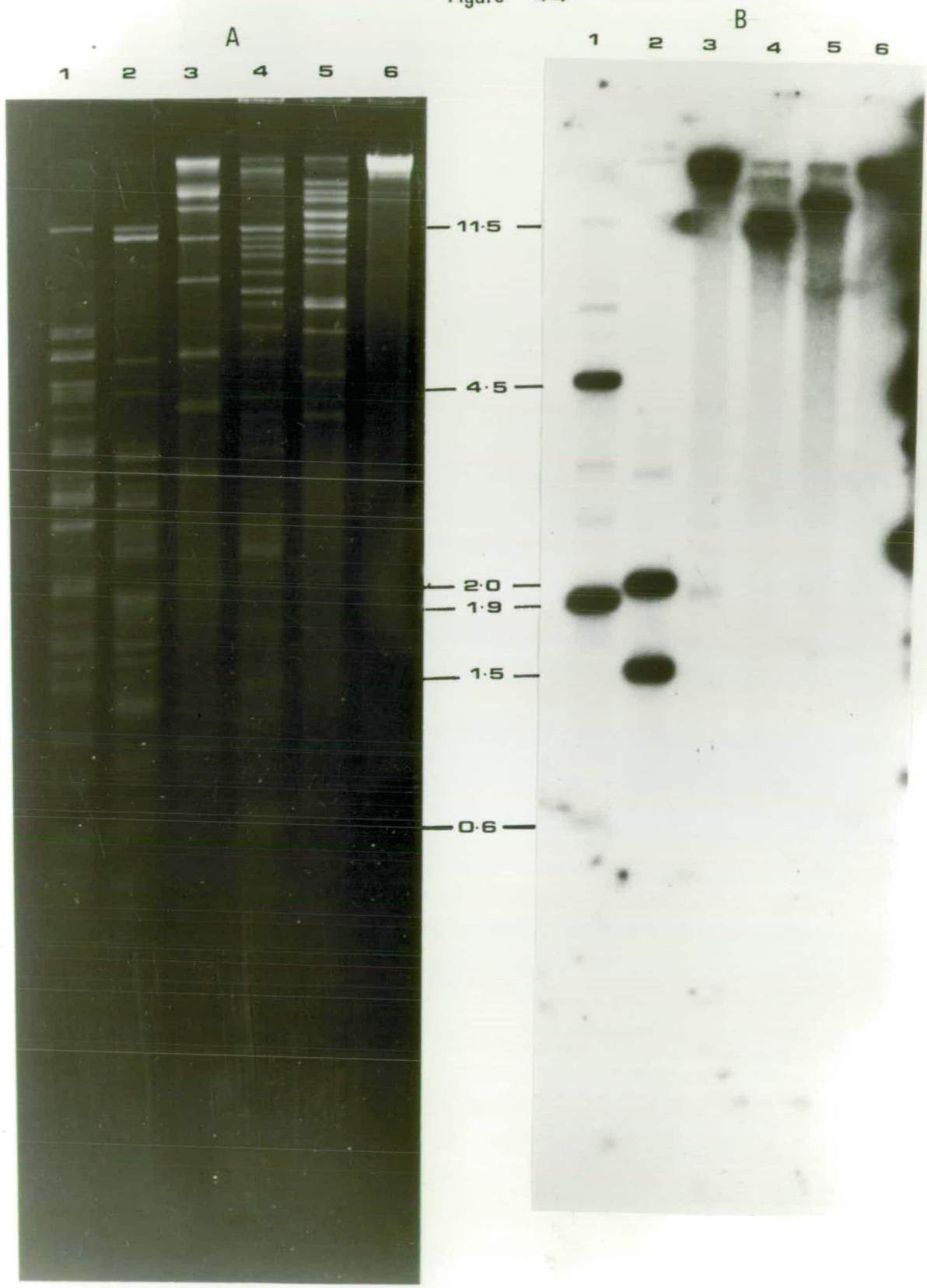


Figure 4.4

Identification of DNA fragments that hybridise to the labelled probe made from λtk-2 DNA. A: ethidium bromide stained 1% agarose gel; B: autoradiograph of a nitrocellulose filter derived from this gel after hybridisation to the labelled probe. Tracks 1-6 are digests of T4 JW819 DNA using 1, R.EcoRI; 2, R.HindIII; 3, R.BglII; 4, R.PstI; 5, R.XhoI; 6, R.KpnI. Estimates of fragment sizes in kb are indicated.

Figure 4·4



two HindIII fragments of T4 DNA of 2.0 and 1.5 kb containing an internal EcoRI fragment of 1.9 kb (Figure A.2). Double digestion of this DNA with R.EcoRI and R.HindIII elucidated the physical map of the λtk recombinants as shown in Figure A.3.

A labelled probe made from the DNA of λtk-2 was hybridised against a nitrocellulose filter carrying T4 DNA digested by various restriction endonucleases, (Figure A.4). Positive hybridisation was seen to two HindIII fragments of 2.0 and 1.5 kb, three EcoRI fragments of 4.5, 1.9 and 0.6 kb, a single PstI fragment of 11.5 kb, the third largest XhoI and KpnI fragments and the largest BglII fragment (Figure A.4). The sizes of these last three fragments cannot be estimated in this experiment because the one per cent agarose gel used to separate the fragments does not allow the accurate estimation of the sizes of DNA fragments above 12 kb.

4. Discussion

A comparison of the physical data presented here with the physical map of the T4 genome constructed by Kutter and O'Farrell (pers. comm.) shows that the third largest XhoI and KpnI fragments, the largest BglII fragment and a PstI fragment of 11.5 kb is in the region between 50 and 60 kb on the physical map (see Figure 1.2), the region which contains the tk gene. The Kutter and O'Farrell map places three contiguous EcoRI fragments of 4.2, 1.9 and 0.7 kb and a HindIII fragment of 1.5 kb, which overlaps the 4.2 and 1.9 kb EcoRI fragments, within this region. This is in good agreement with the data presented here, which indicate that three contiguous EcoRI fragments of 4.5, 1.9 and 0.6 kb and a 1.5 kb HindIII fragment which overlaps the 4.5 and 1.9 kb EcoRI fragments are within the tk region. The

size differences between the two sets of data are probably not significant, but reflect different estimates of the sizes of the same fragments due to the use of different gel systems. The only real discrepancy between these two sets of data, is that Kutter and O'Farrell do not find that their 0.7 kb EcoRI fragment is cut by R.HindIII which would give rise to the 2.0 kb HindIII fragment identified in the λtk recombinants. Presumably this difference indicates that either the T4 DNAs used in this work and by Kutter and O'Farrell differ in the tk region, or that one of the constructed maps is wrong. The fact that the two HindIII fragments described here each contain a single R.EcoRI site, which can give rise to a 1.9 kb EcoRI fragment, and hybridise to only three EcoRI fragments of T4 DNA one of which is 1.9 kb in size, suggests that these two HindIII fragments are contiguous on the T4 chromosome. The mapping method used by Kutter and O'Farrell (pers. comm.) was based on double digestion and two dimensional electrophoresis and so it is conceivable that these authors could have missed the R.HindIII site within the 0.65 kb EcoRI fragment.

The λtk recombinants both carry DNA from the tk region of the T4 genome and have the ability to grow on tdk^- E.coli cells in FUT medium and thus physical and genetic evidence that the T4 tk gene has been cloned is provided. Derivatives of tdk^- cells lysogenic for λtk phages, can grow in FUT medium which suggests that the tk promotor could also be present on the recombinants. The T4 DNA insert in both λtk phages is in the same orientation and a comparison of the physical maps of the tk region presented here by Kutter and O'Farrell, suggest that the tk gene in the λtk recombinants is in the r orientation, assuming that the tk gene is transcribed

anticlockwise on the T4 genetic map, as expected for an early gene (see Wood and Revel, 1976). If the λtk recombinants do carry the tk gene in the r orientation, it is conceivable that expression of the tk gene in lysogens is due to the presence of the weak constitutive rightwards promotor detected in λNM540 by Hopkins et al (1976). Thus it would be desirable to generate λtk phages of l orientation to determine if the tk gene is also expressed in cells lysogenic for these phages. If E.coli tdk$^-$ derivatives lysogenic for a λtk phage of l orientation could grow in FUT medium, then it would seem likely that the tk promotor is also included on the T4 DNA cloned.

The analysis of labelled polypeptides derived from the infection of UV irradiated cells by λtk recombinants and control phages, including the λ vector and wild type, tk amber and tk deletion strains of T4, following separation by polyacrylamide gel electrophoresis, should demonstrate the presence of gptk in the recombinants and the wild type T4 strain, and its absence from the other controls. This analysis should also provide an estimate of the M_r of this polypeptide, which is unknown at present.

More definitive evidence that the λtk recombinants do indeed carry DNA from the tk region of the T4 genome could be obtained by comparing the hybridisation patterns of T4 DNA derived from a strain carrying a point mutation in the tk gene and a strain carrying a deletion of the tk gene, against a labelled probe made from the DNA of a λtk recombinant. Any changes between the hybridisation patterns of the two strains would indicate that the λtk recombinants carry T4 DNA that is missing in the T4 tk deletion strain.

The λtk phages could be examined for the presence of the

neighbouring rI gene by marker rescue analysis using a T4 rI mutant (Edgar et al, 1969). The presence of wild type T4 in lysates derived from mixed infections of λtk phages and a T4 rI mutant, would indicate that marker rescue had occurred and that the rI gene is also carried on the λtk recombinants. This test is complicated by the fact that T4 rI mutants are not conditionally lethal and so any wild type T4 generated could not be selected and would have to be detected on the basis of plaque morphology. The analysis of the polypeptides produced by λtk recombinants, described above, would also reveal the presence of any other T4 specific polypeptides, such as gp rI or the product of the valyl-tRNA synthetase modifying polypeptide, gpvs (Muller and Marchin, 1975). The size of gprI is unknown, but the apparent Mr of gpvs has been estimated as 10,500 (Muller and Marchin, 1975).

The transfer of the two HindIII and the EcoRI fragments of T4 DNA from the λtk recombinants, separately to other λ vectors, would help to localise the position of the tk gene and any other T4 genes present on the cloned fragments (such as the rI or vs genes), within the tk region of the T4 genome. Such phages could be investigated genetically, for example by testing for the presence of the intact tk gene in such derivatives by their ability to grow on a tdk$^-$ host in FUT medium, and biochemically by analysing the T4 specific polypeptides produced by such derivatives. E.coli tdk$^-$ derivatives lysogenic for λT4 phages carrying single DNA fragments from the tk region of the T4 genome, could be investigated for the presence of the supposed tk promotor by their ability to grow on FUT medium.

The λtk recombinant is the only source of cloned T4 DNA between g55 and the tRNA cluster (see Velten and Abelson, 1980) and so would be extremely useful in recovering other fragments from this region of

the T4 genome, which includes the nrdc and e genes which code for the T4 thioredoxin and lysozyme respectively.

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